(54) Title: METHOD OF TRANSCRIPTIONALLY MODULATING GENE EXPRESSION AND OF DISCOVERING CHEMICALS CAPABLE OF FUNCTIONING AS GENE EXPRESSION MODULATORS

(57) Abstract

The present invention provides a method of transcriptionally modulating the expression of a gene-of-interest. The method comprises contacting a cell which is capable of expressing the gene with an amount of a molecule effective to transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene which is expressed by the cell. Molecules useful in the practice of the invention are characterized as follows: (a) do not naturally occur in the cell, (b) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell. Additionally, this invention provides a method for determining whether a molecule known to be a modulator of protein biosynthesis is capable of transcriptionally modulating expression of a gene-of-interest.

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DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

METHOD OF TRANSCRIPTIONALLY MODULATING GENE EXPRESSION
AND OF DISCOVERING CHEMICALS CAPABLE OF FUNCTIONING AS
GENE EXPRESSION MODULATORS

Background of the Invention

Throughout this application, various publications are
referenced by Arabic numerals within parentheses. Full
citations for these publications may be found at the end
of the specification immediately preceding the claims.
The disclosures of these publications in their entireties
are hereby incorporated by reference into this
application in order to more fully describe the state of
the art as known to those skilled therein as of the date
of the invention described and claimed herein.

The expression of a specific gene can be regulated at any
step in the process of producing an active protein.
Modulation of total protein activity may occur via
transcriptional, transcript-processing, translational or
post-translational mechanisms. Transcription may be
modulated by altering the rate of transcriptional
initiation or the progression of RNA polymerase (28).
Transcript-processing may be influenced by circumstances
such as the pattern of RNA splicing, the rate of mRNA
transport to the cytoplasm or mRNA stability. This
invention concerns the use of molecules which act by

modulating the _in vivo_ concentrati n of their target
proteins via regulating gene transcription. The
functional properties of these chemicals are distinct
from previously described molecules which also affect
gene transcription.

Researchers have documented the regulation of
transcription in bacteria by low molecular weight
chemicals (42, 36). Extracellular xenobiotics, amino
acids and sugars have been reported to interact directly
with an intracellular proteinaceous transcriptional
activator or repressor to affect the transcription of
specific genes.

Transcriptional regulation is sufficiently different
between procaryotic and eucaryotic organisms so that a
direct comparison cannot readily be made. Procaryotic
cells lack a distinct membrane bound nuclear compartment.
The structure and organization of procaryotic DNA
elements responsible for initiation of transcription
differ markedly from those of eucaryotic cells.

The eucaryotic transcriptional unit is much more complex
than its procaryotic counterpart and consists of
additional elements which are not found in bacteria.
Eucaryotic transcriptional units include enhancers and
other cis-acting DNA sequences (30, 19). Procaryotic
transcription factors most commonly exhibit a
"helix-turn-helix" motif in the DNA binding domain of the
protein (29, 37). Eucaryotic transcriptional factors
frequently contain a "zinc finger" (37, 12) or a "leucine
zipper" (24) in addition to sometimes possessing the
"helix-turn-helix" motif (26). Furthermore, several
critical mechanisms at the post-transcriptional level
such as RNA splicing and polyadenylation are not found in

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procaryotic systems (21, 35).

In higher eucaryotes, modulation of gene transcription in
response to extracellular factors can be regulated in
both a temporal and tissue specific manner (22). For
example, extracellular factors can exert their effects
by directly or indirectly activating or inhibiting
transcription factors (22, 28).

Modulators of transcription factors involved in direct
regulation of gene expression have been described, and
include those extracellular chemicals entering the cell
passively and binding with high affinity to their
receptor-transcription factors. This class of direct
transcriptional modulators include steroid hormones and
their analogs, thyroid hormones, retinoic acid, vitamin
D₃ and its derivatives, and dioxins, a chemical family of
polycyclic aromatic hydrocarbons (12, 38, 9).

Dioxins are molecules generally known to modulate
transcription, however, dioxins bind to
naturally-occurring receptors which respond normally to
xenobiotic agents via transcriptionally activating the
expression of cytochrome P450, part of an enzyme involved
in detoxification. Similarly, plants also have naturally
occurring receptors to xenobiotics to induce defense
pathways. For example, the fungal pathogen Phytophthora
megasperma induces an anti-fungal compound in soybeans.
Such molecules which bind to the ligand binding domains
of such naturally occurring receptors are not included on
the scope of this invention.

The clinical use of steroid hormones, thyroid hormones,
vitamin D₃ and their analogs demonstrates that agents

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which modulate gene transcription can be used for beneficial effects, although these agents can exhibit significant adverse side effects. Obviously, analogs of these agents could have similar clinical utility as their naturally occurring counterparts by binding to the same ligand binding domain of such receptors.

Indirect transcriptional regulation involves one or more signal transduction mechanisms. The regulation typically involves interaction with a receptor, the receptor being part of a multistep intracellular signaling pathway, the pathway ultimately modulating the activity of nuclear transcription factors. This class of indirect transcriptional modulators include polypeptide growth factors such as platelet-derived growth factor, epidermal growth factor, cyclic nucleotide analogs, and mitogenic tumor promoters (18, 1, 2).

It is well documented that a large number of chemicals, both organic and inorganic, e.g. metal ions, can non-specifically modulate transcription.

Researchers have used nucleotide analogs in methods to modulate transcription. The mechanism involves incorporating nucleotide analogs into nascent mRNA or non-specifically blocking mRNA synthesis. Similarly, researchers have used alkylating agents, e.g. cyclophosphamide, or intercalating agents, e.g. doxorubicin, to non-specifically inhibit transcription.

Moreover, chemical inhibitors of hydroxymethyl-glutaryl CoA reductase, e.g. lovastatin, are known to modulate transcription by indirectly increasing expression of hepatic low density lipoprotein receptors as a consequence of lowered cholesterol levels.

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Signal effector type mol cules such as cyclic AMP, diacylglycerol, and their analogs are known to non-specifically regulate transcription by acting as part of a multistep protein kinase cascade reaction. These signal effector type molecules bind to domains on proteins which are thus subject to normal physiological regulation by low molecular weight ligands (10, 39).

The specific use of sterol regulatory elements from the LDL receptor gene to control expression of a reporter gene has recently been documented in PCT/US88/10095. One aspect of PCT/US88/10095 deals with the use of specific sterol regulatory elements coupled to a reporter as a means to screen for drugs capable of stimulating cells to synthesize the LDL receptor. PCT/US88/10095 describes neither the concept of simultaneously screening large numbers of chemicals against multiple target genes nor the existence of transcriptional modulators which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene-of-interest, and (c) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological effect. The main focus of PCT/US88/10095 is the use of the sterol regulatory elements from the LDL receptor as a means to inhibit expression of toxic recombinant biologicals.

The use of molecules to specifically modulate transcription of a gene-of-interest as described herein has not previously been reported and its use will bring surprise since available literature does not propose the use of a molecule, as described, in a method to

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specifically modulate transcription. Instead, the available literature has reported methods which define domains of transcriptional regulating elements of a gene-of-interest.

Further, the practice of using a reporter gene to analyze nucleotide sequences which regulate transcription of a gene-of-interest is well documented. The demonstrated utility of a reporter gene is in its ability to define domains of transcriptional regulatory elements of a gene-of-interest. Reporter genes which express proteins, e.g. luciferase, are widely utilized in such studies. Luciferases expressed by the North American firefly, _Photinus pyralis_ and the bacterium, _Vibrio fischeri_ were first described as transcriptional reporters in 1985 (8, 11)

A method to define domains of transcriptional regulating elements of a gene-of-interest typically has also involved use of phorbol esters, cyclic nucleotide analogs, concanavalin A, or steroids, molecules which are commonly known as transcriptional modulators. However, available literature shows that researchers have not considered using a transcription screen to identify specific transcriptional modulators. Apparently, success would be unlikely in doing so, however, we have demonstrated herein that this is not the case.

There is utility in developing the method of transcriptional modulation of a gene-of-interest by using such molecule as described herein. This method will allow the development of novel pharmaceuticals and circumvent many of the problems associated with the therapeutic use of recombinant biological factors.

Problems associated with the therapeutic use of

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recombinant biological factors includ the technical
difficulties of large scale protein purification, the
high costs of protein production, the limited shelf-life
of most proteins and in some cases a short biological
half-life of the administered protein in the organism.
Additionally, therapeutic delivery of proteins normally
requires injection and frequently induces an immune
reaction in situations where chronic administration is
required. The method described herein provides a means
of up-regulating the expression of proteins, e.g.
membrane receptors, which are not readily amenable to
administration as injectable biologicals.

Furthermore, chemical molecules specifically regulating
the activity of one member of a group of closely related
proteins are difficult to produce. Molecules,
structurally related at the protein level, may possess
distinct regulatory elements at the DNA level which
control their expression. Thus, molecules such as the
chemical transcriptional modulators defined herein can
provide a greater opportunity for specifically modulating
the activity of structurally related proteins. One
example is the ras oncogene family, where the H-, N- and
K-ras proteins are highly related but wherein the three
genes have distinct structures.

Finally, the molecules described herein may also serve to
mimic normal physiological response mechanisms, typically
involving the coordinated expression of one or more
groups of functionally related genes. Therefore,
determining whether a molecule can specifically
transcriptionally modulate the expression of a gene-of-
interest and the ultimate clinical use of the molecule
provides a therapeutic advantage over the use of single
recombinant biologicals, or drugs which bind directly to
the final target protein encoded by the gene-of-interest.

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Summary of the Invention

The present invention provides a method of transcriptionally modulating the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. The method comprises contacting a cell which is capable of expressing the gene with an amount of a molecule effective to transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene which is expressed by the cell.

Molecules useful in the practice of the invention are characterized as follows (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene-of-interest, and (c) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological or pathological effect.

Additionally, this invention provides a method of determining whether a molecule, not previously known to be a modulator of protein biosynthesis, is capable of transcriptionally modulating the expression of a gene-of-interest. The method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each such cell comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a reporter gene which expresses a
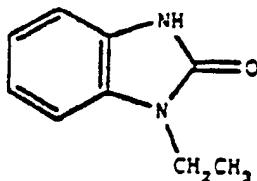
polypeptide capable f producing a detectable signal, coupled to, and under the control of, the promoter, and the contacting is carried out under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measurable detectable signal to be produced by the polypeptide expressed by the reporter gene and the amount of the signal produced may be quantitatively determined. The amount of produced signal so determined is compared with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to thereby identify the molecule as one which causes a change in the detectable signal produced by the polypeptide expressed by the reporter gene and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene-of-interest.

The present invention still further provides a method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect in the organism. The method comprises administering to the organism an amount of a molecule effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. A molecule useful in the method: (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene-of-interest, and (c) binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the organism, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological or

pathological effect.

This invention provides a method for enhancing the
expression of human growth hormone by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, human growth hormone. The method comprises
contacting the cell with an amount of a molecule having
the structure:

effective to enhance the expression of human growth
hormone by the cell.

Additionally, this invention provides a method of
increasing the growth of a human being who (i)
comprises DNA encoding, and (ii) is capable of
expressing, human growth hormone, comprising
administering to the human being an amount of a
molecule having the structure:

effective to enhance expression of human growth hormone
by, and thus growth of, the human being.

The invention also provides a method for enhancing the
expression of human growth hormone by a cell which (i)
comprises DNA encoding, and (ii) is capable of

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expressing, human growth hormon , c mprising contacting
the cell with an amount of a molecule having the
structur :

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effective to enhance the expression of human growth
hormone by the cell.

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Another provision of the present invention concerns a
method of increasing the growth of a human being who
(i) comprises DNA encoding, and (ii) is capable of
expressing, human growth hormone. The method comprises
administering to the human being an amount of a
molecule having the structure:

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effective to enhance expression of human growth hormone
by, and thus growth of, the human being.

This invention also concerns a method for enhancing the
35 expression of human growth hormone by a cell which (i)

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comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:
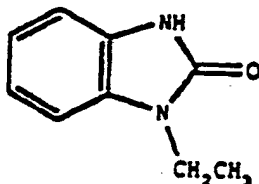
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$$SO_3H$$

HO   NH₂

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effective to enhance the expression of human growth hormone by the cell.

Further, this invention provides a method of increasing

15   the growth of a human being who_ (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:
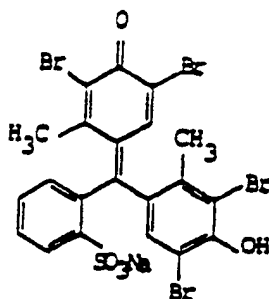
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$$SO_3H$$

HO   NH₂

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effective to enhance expression of human growth hormone by, and thus growth of, the human being.

30   Additionally, this invention concerns a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having
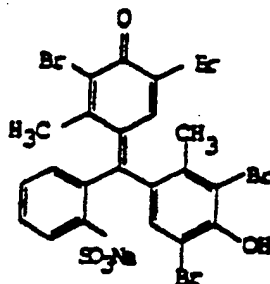
35   the structure:

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effective to enhance the expression of human growth hormone by the cell.

Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:

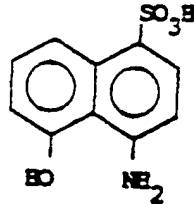effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Further, the invention provides another method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:

wherein R is $-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-NHCH_2CH_2CH_2\overset{CH_3}{\underset{CH_3}{\overset{|}{\underset{|}{N^+}}}}CH_3$ or $-\overset{-}{O}-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-OCH_3,$
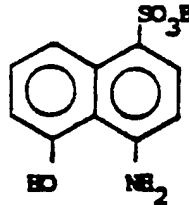
effective to enhance the expression of human growth hormone by the cell.

Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:
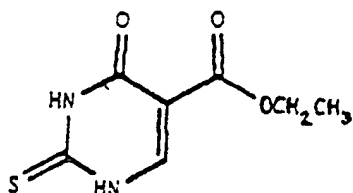
wherein R is $-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}-NHCH_2CH_2CH_2\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle CH_3}{|}}{N+}}CH_3$ or $-\,^-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}-OCH_3,$

effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Further, this invention provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:

effective to enhance the expression of human growth hormone by the cell.

Further more, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:

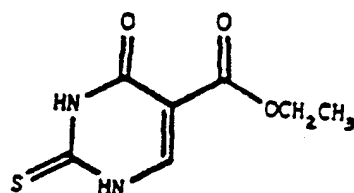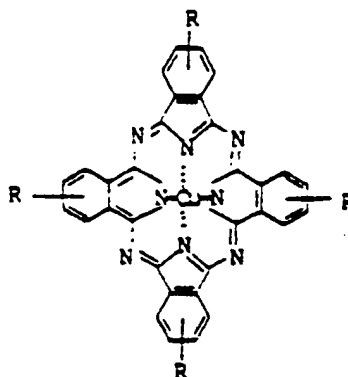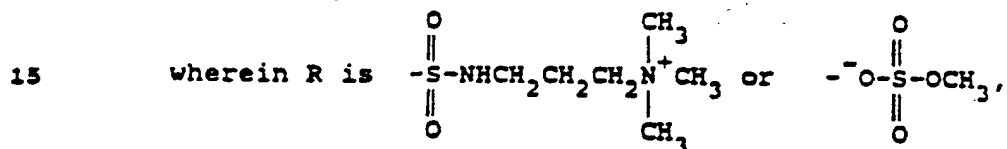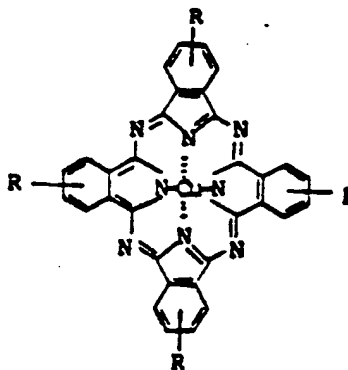effective to enhance expression of human growth hormone by, and thus growth of, the human being.

The present invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

effective to enhance the expression of G-CSF by the cell.

The present invention also provides a method for increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:

effective to enhance the expression of GCSF by, and thus increase the formation of neutrophils in, the human being.

The present invention also provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
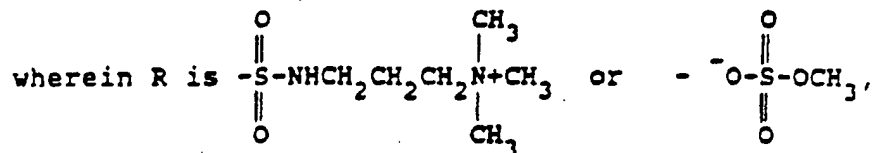
effective to enhance the expression of G-CSF by the cell.

Additionally, the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:
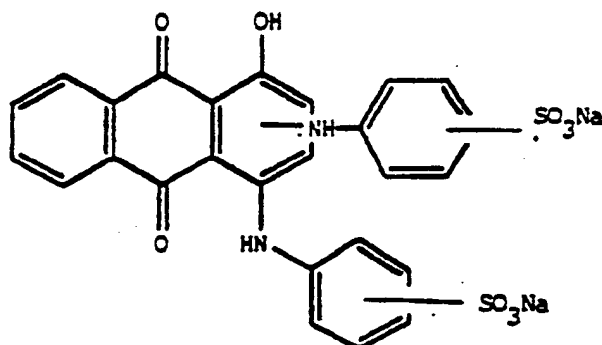
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H₃C, N
O
CH₃

NH₂

effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

Further, the invention provides another method for
enhancing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
cell with an amount of a molecule having the structure:

H₃C, N⁺, CH₃

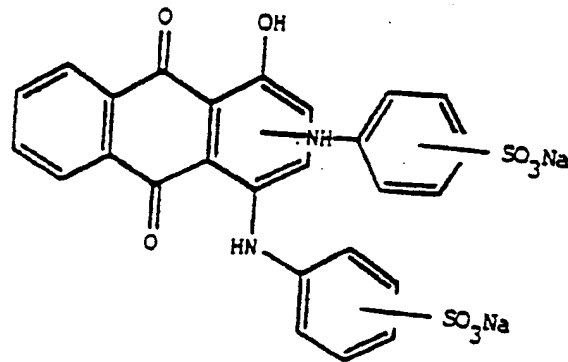H₃C, N, CH₃
H₃C, CH₃

effective to enhance the expression of G-CSF by the
cell.

The present invention provides another method of
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF. The method comprises
administering to the human being an amount of a
molecule having th structure:

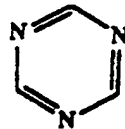effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

The invention also concerns a method for enhancing the
expression of G-CSF by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF
comprising contacting the cell with an amount of a
molecule having the structure:

effective to enhance the expression of G-CSF by the
cell.

Additionally, the invention concerns a method of
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising administering
to the human being an amount of a molecule having the
structur :

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effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

This invention further concerns a method for enhancing the expression of G-CSF by a cell-which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
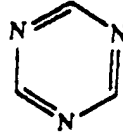
effective to enhance the expression of G-CSF by the cell.

Further the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

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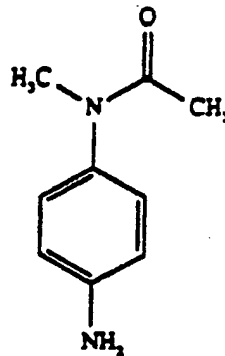effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

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Additionally, the present invention concerns a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising contacting the cell with an amount of a molecule having the structure:
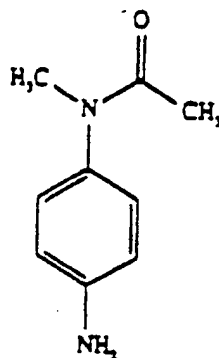
$$NH_2$$
$$O = S = O$$

$$CH_2NH_2$$

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effective to enhance the expression of G-CSF by the cell.

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The invention further concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF wherein the method comprises administering to the human being an amount of a molecule having the structure:

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$$O = \underset{\underset{}{\overset{NH_2}{|}}}{S} = O$$
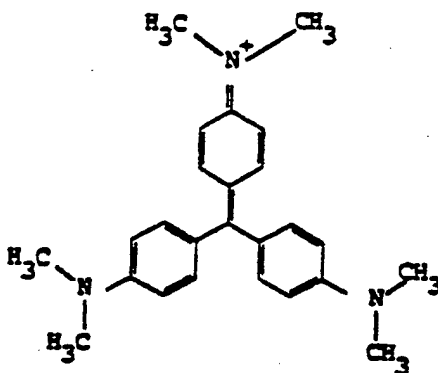
(benzene ring with CH₂NH₂ substituent)

CH₂NH₂

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Also, the invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
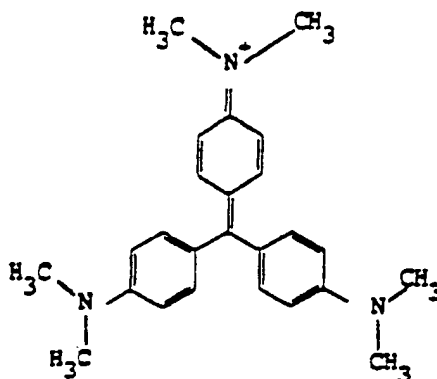
Br

(benzene ring with Cl substituents)

Cl   Cl

effective to enhance the expression of G-CSF by the cell.

The invention additionally concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, wherein the method comprises administering to the human being an amount of a molecule having the structure:

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effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

Another provision of the present invention is a method
for enhancing the expression of G-CSF by a cell which
(i) comprises DNA encoding, and (ii) is capable of
expressing G-CSF. The method comprises contacting the
cell with an amount of a molecule having the structure:

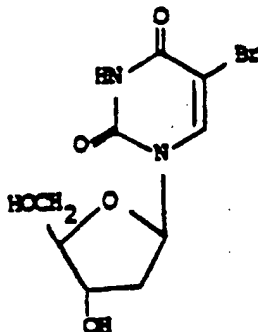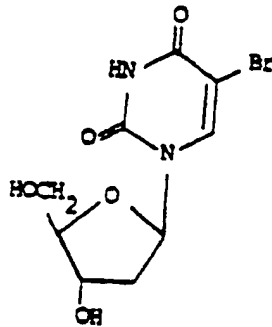effective to enhance the expression of G-CSF by the
cell.

Additionally, the invention provides a method of
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing G-CSF. The method comprises
administering to the human being an amount of a
molecule having the structure:

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effective to enhance expression of G-CSF by, and thus increase the formation of n utrophils in the human being.

The present invention provides another method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

effective to enhance the expression of G-CSF by the cell.

Further, the present invention concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:
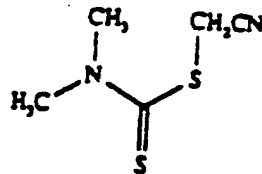
effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

This invention also concerns a method of decreasing the
expression of G-CSF by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
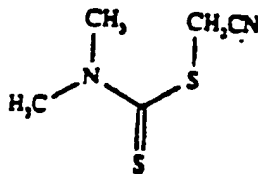of a molecule having the structure:

effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises

DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
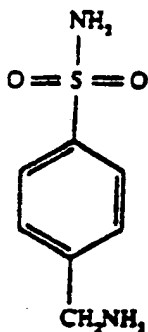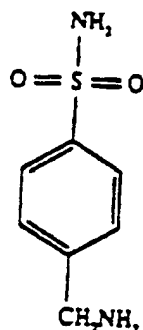
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effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

15   Further, the invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

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effective to decrease the expression of G-CSF by the cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
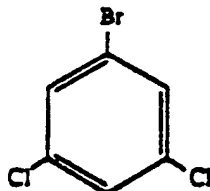

effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

The invention further provides a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
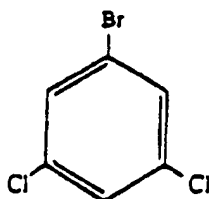
effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and effecting
the metabolic functions of neutrophils, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
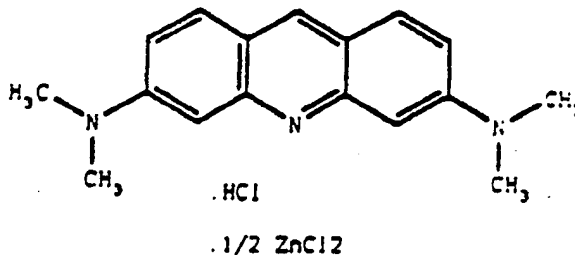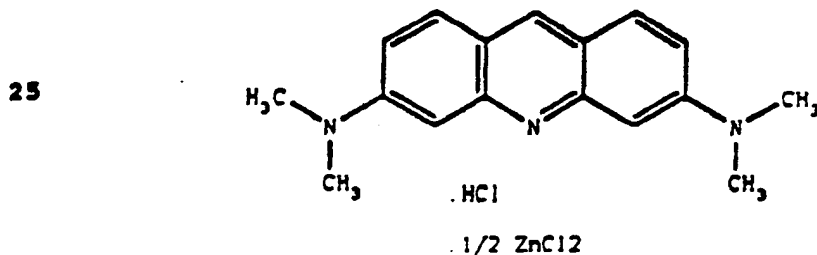of a molecule having the structure:

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

Further, the present invention a method of decreasing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
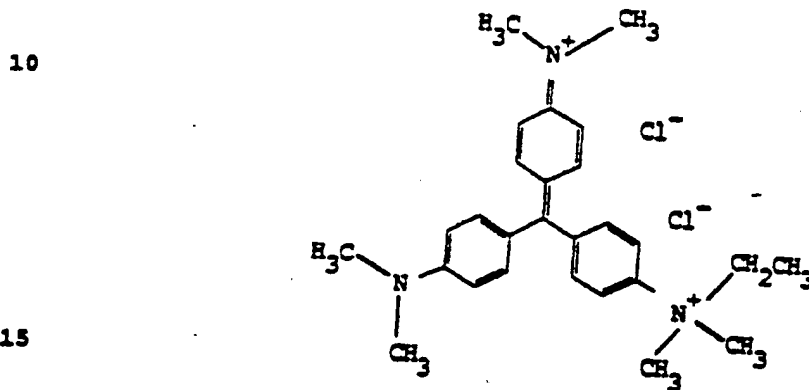of a molecule having the structure:

$$CH_2OH$$

effective to decrease the expression of G-CSF by the cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

$$CH_2OH$$

effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.
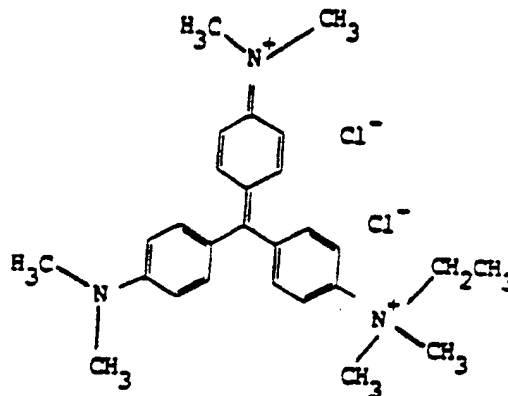
The present invention further provides a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of

expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

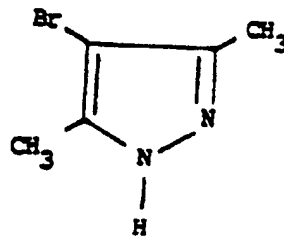effective to decrease the expression of G-CSF by the. cell.

Further still the invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

effective to decrease expression of G-CSF by, and thus decreas the formation and affect the metabolic

functions of neutrophils in, the human being.

The present invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
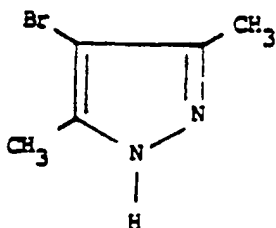
$$
\begin{array}{c}
\text{CONH}_2 \\
\text{OH}
\end{array}
$$

effective to decrease the expression of G-CSF by the cell.

Additionally, the present invention concerns a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
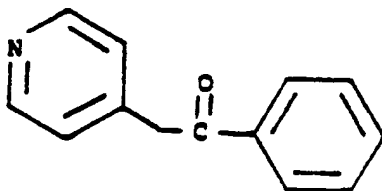
$$
\begin{array}{c}
\text{CONH}_2 \\
\text{OH}
\end{array}
$$

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

The present invention also concerns a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
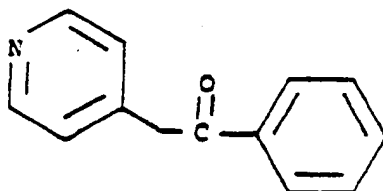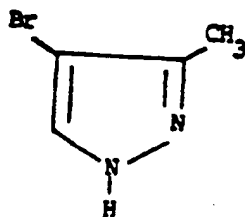cell with an amount of a molecule having the structure:

effective to decrease the expression of G-CSF by the
cell.

Further, the invention concerns a method of decreasing
the formation of neutrophils and affecting the
metabolic functions of neutrophils, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:

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effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

Moreover, the invention provides a method of decreasing
the expression of a mammary tumor virus by a cell which
(i) comprises DNA encoding, and (ii) is capable of
expressing, a mammary tumor virus. The method
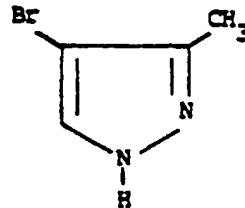comprises contacting the cell with an amount of a
molecule having the structure:

effective to decrease the expression of a mammary tumor
virus by the cell.

Finally, the invention also provides a method of
suppressing the proliferation of a mammary tumor virus
in a subject who (i) comprises DNA encoding, and (ii)
is capable of expressing, a mammary tumor virus,

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comprising administering to the subject an amount of a molecule having the structure:

effective to suppress the proliferation of a mammary tumor virus in the subject.

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Brief D scripti n f the Figures

Figure 1 is a partial restriction enzyme cleavage map of
the plasmid pD0432 which contains the luciferase gene
from the firefly, _Photinus pyralis_.

Figure 2 is a partial restriction enzyme cleavage map of
the plasmid pSVLuci which contains the luciferase gene
from the firefly, _Photinus pyralis_.

Figure 3 is a partial restriction enzyme cleavage map of
the plasmid pMLuci which contains the luciferase gene of
the firefly, _Photinus pyralis_ and the mouse mammary tumor
virus long terminal repeat.

Figure 4 is a partial restriction enzyme cleavage map of
the plasmid pUXLuci which contains the luciferase gene
from the firefly, _Photinus pyralis_.

Figure 5 is a partial restriction enzyme cleavage map of
the plasmid phGH:CAT which contains the CAT gene and
human growth hormone promoter sequences.

Figure 6 is a partial restriction enzyme cleavage map of
the plasmid phGH-Luci which contains the luciferase gene
from the firefly, _Photinus pyralis_ and human growth
hormone promoter sequences.

Figure 7 is a partial restriction enzyme cleavage map of
the plasmid pJM710 which contains G-CSF upstream
sequences.

Figure 8 is a partial restriction enzyme cleavage map of
the plasmid pGEM5-Luci which contains the luciferase gene
from the firefly, _Photinus pyralis_.

Figure 9 is a partial restriction enzyme cleavage map of
the plasmid PG-Luc 1 which contains both the luciferase
gene from the firefly, _Photinus pyralis_, and G-CSF
upstream sequences.

Figure 10 is a bar graph illustrating induction of
luciferase expression in reporter cell lines containing
MMTV (M10), human growth hormone (532), and human G-CSF
(G21) promoter sequences, in response to known
transcriptional inducers.

Figure 11 is a bar graph illustrating the effect of
steroids on luciferase expression in the MMTV reporter
cell line M10.

Figure 12 is a bar graph illustrating specific induction
of luciferase expression in reporter cell lines for MMTV
(M10), human growth hormone (532) and human G-CSF (G21)
promoters in response to chemicals identified in a high
throughput screen and known transcriptional inducers.

Figure 13 is a bar graph illustrating specific inhibition
of luciferase expression in reportor cell lines for MMTV
(M10), human growth hormone (532), and human G-CSF (G21)
in response to chemicals identified in a high throughput
screen.

Figure 14 is an autoradiograph of a Northern blot
illustrating increased G-CSF mRNA production by the human
epithelial cell line U5637 in response to chemicals #670
and #1255 and IFN-gamma as compared to the solvent DMSO.
Reprobing with beta-actin was used to normalize for the
amount of mRNA that had been loaded onto the gel.

Figure 15 is an autoradiograph of a polyacrylamide gel

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illustrating an S1 nucleas pr tection analysis of
increased mRNA producti n by th human bladder carcinoma
cell line 5637 in response to lead chemicals #542, #1255,
#1793 and #1904. "RNA" indicates the sources of the RNA
preparations used in individual lanes. "Probe" indicates
the mRNA-specificities of probes used in individual
lanes. "Compound" lists the compounds with which the 5637
cells were treated prior to RNA extraction and loading on
individual gel lanes ("Cyclo" means cycloheximide).
"Conc" indicates three different compound concentrations
used in the experiment (L = low, M = medium, H = high).
G, GM and A indicate the correct sizes of G-CSF-, GM-CSF-
and Actin-specific nuclease-protected mRNA/Probe hybrids.

Figure 16 illustrates a dose response analysis of
chemicals #80, #670, and #1780 using the G-CSF reporter
cell line G21. The amount of luciferase expression is
indicated in arbitrary units.

Figure 17 is a bar graph illustrating increased G-CSF
secretion by 5637 cells treated for 48 hours in serum-
containing media with the samples indicated on the
abscissa. TNF-alpha was used at 5 ng/ml. Chemicals #542
and #1780 were used at 50 uM or 1 uM and 0.2 uM final
concentration, respectively. Both chemicals were used in
DMSO at a final concentration of 0.5 %. The ordinate
indicates the concentration of G-CSF secreted into 5 ml
of serum-containing media by 25 square cm of confluent
5637 cells.

Figure 18 illustrates a dose response analysis of
chemical #542 using the G-CSF reporter cell line G 21
(solid line) and the MTT respiratory inhibition
cytotoxicity assay (dotted line). Respiratory inhibition
in percent of untreated control cells (Ordinate, left

scale) and luciferase expression of #542- treated over
solvent-treated cells (ordinate, right scale) are plotted
against #542 concentration (abscissa).

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Detailed Description f the Invention

The present invention provides a method of
transcriptionally modulating the expression of a
homologous gene-of-interest, the expression of which is
associated with a defined physiological or pathological
effect within a multicellular organism. The method
comprises contacting a cell which is capable of
expressing the gene with an amount of a molecule
effective to transcriptionally modulate the expression of
the gene. Modulating the expression of the gene affects
the level of the protein encoded by the gene which is
expressed by the cell. As used herein a homologous gene-
of-interest means a gene which is naturally associated
with an organism or virus encoding and expressing the
gene. Therefore, this definition would not include a
synthetic or recombinant gene constructed by genetic
engineering methods so as to position the gene-of-
interest under the control of a heterologous promoter.

The term "physiological effect" as used herein is defined
as an effect characteristic of, or appropriate to, an
organism's healthy or normal functioning. Further, the
term "pathological effect" as used herein is defined as
an effect altered or caused by disease.

Molecules useful in the practice of the invention are
characterized as follows (a) do not naturally occur in
the cell, (b) specifically transcriptionally modulate
expression of the gene-of-interest, and (c) bind to DNA
or RNA or bind to a protein through a domain of such
protein which is not a ligand binding domain of a
receptor which naturally occurs in the cell, the binding
of a ligand to which ligand binding domain is normally

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associated with the defined physiological or pathological effect.

In one example of this invention, the molecule would not occur naturally in any cell of a higher eucaryotic organism. In another example of this invention the molecule would not occur naturally in any cell, e.g. a mineral. In yet another example, the molecule would not occur naturally, e.g. a synthetic molecule.

The phrase "specifically transcriptionally modulate expression of the gene-of-interest" as used herein means modulating the expression of the gene-of-interest without modulating the expression of other genes in the cell in a way which would cause an adverse effect on (a) an organism containing the cell in the case where the cell is within the organism or (b) the growth or the culturing of the cell, in the case where the cell is being grown or cultured to make a product where the amount of product produced is associated with expression of the gene-of-interest. However, within this definition where the drug is used to treat, for example, parasitic infection, drug application is intended to cause an adverse effect on the cells of the parasite (which may contain the gene-of-interest), but not on the cells of the host organism. In this context, a gene-of-interest may constitute a single gene or a limited number of genes whose expression can be functionally coordinated. One example of coordinate gene regulation is exhibited by the physiological growth modulators termed the transforming growth factor-β family of polypeptides (TGF-βs) (90). TGF-βs control the extracellular matrix (ECM) by; (1) increasing expression of the genes encoding ECM polypeptides, e.g. collagen, fibronectin and osteopontin; (2) increasing expression of receptors for the ECM, e.g.

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the integrins; and (3) increasing expression of protease inhibitors (e.g. TIMP and PAI-I) while decreasing expression of secreted proteases (e.g. collagenase and stromelysin). This coordinate regulation may allow TGF-βs to be useful in the repair of surface wounds, cartilage and bone. Molecules with properties as described in this invention, with lower molecular weights than TGF-β, and which can be either chemically synthesized or readily isolated from natural sources, yet mimic the coordinate regulation of extracellular matrix as induced by the TGF-βs, have significant advantages as therapeutic agents over the use of such complex polypeptides.

Moreover, the phrase "transcriptionally modulate the gene-of-interest" infers a notion of directness. Thus, as used herein, "transcriptionally modulate expression of a gene-of-interest" by a molecule means the effect upon transcription of the gene resulting from either (a) direct binding of the molecule to DNA or RNA, a DNA- or RNA-binding protein, and/or a DNA- or RNA- binding protein complex, or (b) direct binding of the molecule to a protein which directly chemically modifies a DNA- or RNA- binding protein or protein complex.

As used herein "chemically modifies" a DNA- or RNA-binding protein or protein complex means to modify the protein or protein complex through a chemical reaction, including but not limited to, phosphorylation, glycosylation, methylation, acetylation, adenoribosylation, acylation, myristylation, reduction, oxidation, covalent oligomerization or polymerization or proteolytic cleavage.

The invention provides a cell capable of expressing the

gene-of-interest, the expression of which is associated
with a defined physiological or pathological effect
within a multicellular organism. The cell may be a human
cell, an animal cell, a plant cell or any eucaryotic cell
or procaryotic cell from whatever source.

Further, in the practice of the invention, the gene-of-
interest whose expression is associated with a defined
physiological or pathological effect within a
multicellular organism, may be a human gene.

Moreover, the gene-of-interest may encode a hematopoietic
protein. Hematopoietic proteins may include, but are not
limited to, colony stimulating factors and erythropoietin
(EPO).

Examples of colony stimulating factors useful in the
practice of this invention include, but are not limited
to, granulocyte-macrophage colony stimulating factor
(GM-CSF), granulocyte colony stimulating factor (G-CSF),
and macrophage colony stimulating factor (M-CSF).

Further, the gene-of-interest of the invention may encode
an interleukin (IL) or a cytokine, or a growth modulating
factor. One example of such a growth modulating factor
would be a member of the transforming growth factor-ß
(TGF-ß) family i.e. TGF-ß1 or TGF-ß2 or TGF-ß3. A gene-
of-interest may also encode a receptor for a steroid
hormone, such as the testosterone receptor or the
estrogen receptor or a receptor for a TGF-ß.

The gene-of-interest may also encode a growth hormone.
Examples of growth hormones include, but are not limited
to, human, bovine, porcine, avian, ovine, piscine, and

equin growth horm nes. Additionally, the gene-of-
interest may also ncode polypeptide analogs of th
above-identified growth hormones. Additionally, a gene-
of-interest may encode a growth hormone releasing factor.

The present invention also provides a viral gene as the
gene-of-interest. The viral gene may be a retroviral
gene. Retroviral genes of the invention may be from the
HIV, HTLV-1, or HTLV-2 virus.

In the practice of the invention the viral gene may be a
gene from a hepatitis virus, a herpes virus, a papilloma
virus, a cytomegalovirus, or an animal virus.

Animal viruses of the invention may include, but are not
limited to, pseudorabies, Marek's, Newcastle's Disease,
and IBR viruses.

The gene-of-interest, whose expression is associated with
a defined physiological or pathological effect within a
multicellular organism, may also be a plant gene. The
plant gene may encode an agronomically important trait.
Examples of agronomically important traits may include,
but are not limited to, germination, sprouting,
flowering, fruit ripening, salt tolerance, herbicide
resistance, pesticide resistance, fungicide resistance,
temperature resistance, and growth.

Additionally, in the practice of the invention the gene-
of-interest may be a protozoan gene. Examples of
protozoans may include, but are not limited to, a
selection from the group consisting of Trypanosoma,
Plasmodium, Leishmania, Giardia, Entamoeba, Toxoplasma,
Babesia, and Cryptosporidiosis.

Moreover, the gene-of-interest whose expr ssion is
associated with a defined physiological or pathological
effect within a multicellular organism, may be a helminth
gene.

Further, the gene-of-interest may also be an oncogene.
Examples of oncogenes may include, but are not limited
to, the phl-abl oncogene, the neu oncogene, or the src
oncogene. Additionally, the oncogene may be selected
from the group consisting of H-ras, N-ras, and K-ras
oncogenes.

The present invention additionally provides that the
gene-of-interest, whose expression is associated with a
defined physiological or pathological effect within a
multicellular organism, may encode a naturally occurring
receptor. The naturally occurring receptor may be the
human low density lipoprotein (LDL) receptor. Further,
the receptor may be the receptor for a hemopoietic
protein. Examples of hematopoietic proteins may include,
but are not limited to, a selection from the group
consisting of M-CSF, G-CSF, GM-CSF, and EPO.

The naturally occurring receptor encoded by the gene-of-
interest may also be the receptor for an interleukin
(IL). Examples of an IL may include, but are not limited
to, a selection from the group consisting of IL-1, IL-2,
IL-3, IL-4, IL-5, IL-6, IL-7 and IL-8.

Additionally, in the practice of the invention, the
naturally occurring receptor may be a cell surface
protein which mediates infection of the cell by a virus.
Examples of viruses may include, but are not limited to,
HIV, HTLV-1, HTLV-2, a hepatitis virus, a herpes virus,
a papilloma virus, a cytomegalovirus and a rhinovirus.

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In one example of the invention, the receptor which
naturally occurs in the cell is a testosterone receptor.
In another example of the invention, the receptor which
naturally occurs in the cell is an estrogen receptor.

Typically, a ligand, in the context of this invention, is
a molecule with a molecular weight of less than 5,000
daltons, more typically less than 2,000 daltons.

This invention also provides a method of determining
whether a molecule not previously known to be a modulator
of protein biosynthesis is capable of transcriptionally
modulating the expression of a gene-of-interest. The
method comprises contacting a sample which contains a
predefined number of cells with a predetermined amount of
a molecule to be tested. Each cell comprises DNA
consisting   essentially   of   (i)   a   modulatable
transcriptional regulatory sequence of the gene-of-
interest, (ii) a promoter of the gene-of-interest, and
(iii) a reporter gene   which expresses a polypeptide
capable of producing a detectable signal, coupled to, and
under the control of, the promoter. The polypeptide
expressed by the reporter gene produces the detectable
signal under conditions such that the molecule, if
capable of acting as a transcriptional modulator of the
gene-of-interest, causes a measurable detectable signal
to be produced by the polypeptide expressed by the
reporter gene.

Quantitatively determining the amount of the signal
produced requires comparing the amount of signal produced
compared to the amount of produced signal detected in
the absence of any molecule being tested or upon
contacting the sample with any other molecule.   The

comparison permits the identification of the molecule as one which causes a change in the detectable signal produced by the polypeptide expressed by the reporter gene and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene-of-interest.

The phrase "a modulatable transcriptional regulatory sequence of a gene-of-interest" as used herein concerns a DNA sequence capable of regulating the initiation of transcription from the promoter of the gene-of-interest.

Molecules useful in the practice of this invention have the following characteristics. The molecule does not naturally occur in the cell. The molecule specifically transcriptionally modulates expression of the gene-of-interest. Further, the molecule binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell. The binding of a ligand to the ligand binding domain is normally associated with the defined physiological or pathological effect.

The term "promoter" of a gene-of-interest is defined herein as a minimal DNA sequence necessary for specific initiation of transcription.

In the practice of the invention the sample may comprise cells in monolayers or cells in suspension. The cells of the invention may comprise human, animal, or plant cells. In one example of the invention, the cells are bacterial cells. In another example of the invention, the cells are fungal cells.

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Further, the invention provides that the predefined
number of cells contained in the sample may be from about
2×10^2 to about 5×10^5 cells, typically from about 10^3
to about 5×10^4 cells. In theory, the ideal method
might use only one cell. In practice, the method may
include at least 10 cells per samples. More practically
the method may include at least 100 cells per sample.

The invention also provides that the predetermined amount
of the molecule to be tested may be based on the volume
of the sample. Further, the predetermined amount of the
molecule to be tested may be from about 1pM to about
500μM. Typically, in a primary highthroughtput screen
the predetermined amount would be from about 10nM to
about 500μM. Typically, in a secondary screen to
evaluate initial lead compounds, the predetermined amount
·would be from about 1pM to about 20 μM.

Further, the invention provides that contacting a sample
which contains a predefined number of cells with a
predetermined amount of a molecule to be tested may be
effected from about 1 to about 24 hours, typically from
about 2 to about 12 hours. Moreover, contacting a sample
which contains a predefined number of cells with a
predetermined amount of a molecule to be tested may be
effected with more than one predetermined amount of the
molecule to be tested. The molecule to be tested may be,
but is not limited to, a purified molecule. As used
herein, the molecule may also be a mixture of different
molecules, e.g. in a fermentation broth or in a natural
product extract. Typically, a fermentation broth would be
produced by fermentation of a suitable sample under
standard conditions known to those skilled in the art.
Examples of natural product extracts would include, but
are not restricted to, plant extracts, seaweed extracts,

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insect extracts, spider venoms, etc.

A minimal modulatable transcriptional regulatory sequence
may comprise a few nucleotides in length, such as the so-
called CCAAT box motif, the octamer binding motif or the
heat shock element (91). A gene-of-interest may typically
possess multiple motifs with different combinations of
modulatable transcriptional regulatory sequences. It has
been shown that enhancer elements or other regulatory
motifs can be present at many kilobases upstream or
downstream from the transcription start site of a gene-
of-interest. Thus, as used herein, a modulatable
transcriptional regulatory sequence may comprise a few
nucleotides or may be naturally or synthetically
constructed having multiple elements spanning several
hundred kilobases in length. Transcription can also be
influenced by the particular nuclear location or local
chromatin configuration. In order to optimize a screen
for substances that modulate regulatory elements, it may
be preferable to situate a reporter gene at the exact
chromosomal region where the gene-of-interest resides
normally. However, it would be clear to one skilled in
the art that the reporter gene may be situated anywhere
in a chromosome. In one example in the invention, the
modulatable transcriptional regulatory sequence comprises
a cloned genomic regulatory sequence. In another example
of the invention, the DNA consists essentially of more
than one modulatable transcriptional regulatory sequence.

A series of experiments described by K.R. Thomas and M.R.
Capecchi (44,45,46) have shown that it is possible to
direct transfected or microinjected DNA, using homologous
DNA sequences, to a specific chromosomal location in
mammalian cells. Using homologous recombination, it is
possible to replace a gene-of-interest with other

sequences at a high frequency. The materials required
include a reporter gene (such as luciferase), a
selectable marker (such as the neomycin
phosphotransferase II gene from Tn5), and sequences
5   derived from the gene-of-interest. These materials would
be used to construct a vector which would include, in one
embodiment of this invention, a fusion consisting of the
gene-of-interest (free of its normal upstream promoter
sequences) and the reporter gene where the reporter gene
10   product is still active.

Two types of selectable markers, one positive and one
negative, such as the Herpes Simplex thymidine kinase
(TK), greatly enhance the likelihood of achieving
15   homologous recombination. Use of one copy of the TK gene
located outside the homologous regions at either end
allows for selection of "correct" homologous
recombinants. Neomycin resistant cells which are
recombined randomly will usually insert via the ends of
20   the linearized, transfected DNA and thus include the TK
gene(s). This will allow for selection against these
cells in the presence of gancyclovir which is converted
to a toxic product. This is the method specifically
described by Capecchi. However, this method has not been
25   used to produce cell lines to screen for compounds which
modulate the expression of a target gene-of-interest as
described herein.

The invention provides that the reporter gene in the DNA
30   contained in the cell sample, which expresses a
polypeptide capable of producing a detectable signal
coupled to, and under control of, the promoter, may be
inserted downstream of the endogenous promoter of the
gene-of-interest by homologous recombination. The
35   following provides a method of determining whether a

molecul not previously known to be a modulator of
protein biosynthesis is capable of transcriptionally
modulating the expression of a gene-of-interest which
comprises contacting a sample which contains a predefined
number of cells with a predetermined amount of a molecule
to be tested, each such cell comprising DNA consisting
essentially of (i) a modulatable transcriptional
regulatory sequence of the gene-of-interest, (ii) a
promoter of the gene-of-interest, and (iii) a DNA
sequence transcribable into mRNA coupled to and under the
control of, the promoter, under conditions such that the
molecule, if capable of acting as a transcriptional
modulator of the gene-of-interest, causes a measurable
difference in the amount of mRNA transcribed from the DNA
sequence, quantitatively determining the amount of the
mRNA produced, comparing the amount so determined with
the amount of mRNA detected in the absence of any
molecule being tested or upon contacting the sample with
any other molecule, and thereby identifying the molecule
as one which causes a change in the amount of detectable
mRNA, and thus identifying the molecule as a molecule
capable of transcriptionally modulating the expression of
the gene-of-interest.   In one example of the above-
described method, the molecule (a) does not naturally
occur in the cell, (b) specifically transcriptionally
modulates expression of the gene-of-interest, and (c)
binds to DNA or RNA or binds to a protein through a
domain of such protein which is not a ligand binding
domain of a receptor which naturally occurs in the cell,
the binding of a ligand to which ligand binding domain
is normally associated with a defined physiological or
pathological effect.

Modulatable transcriptional regulatory sequences may also

occur within introns of a gen -of-interest. Typically,
the exact location of all such regulatory sequences of a
gene-of-interest will be unknown. In one example of this
invention, the reporter gene will be a reporter gene
which can be transcribed into mRNA which can be
quantified as a detectable signal. This approach would
allow (i) allow all the modulatable transcriptional
regulatory sequences of the gene to be in their native
environment and arrangement relative to the gene-of-
interest including any and all transcriptional regulatory
sequences within introns, (ii) obviate the necessity of
using permanent cell lines to construct reporter cell
lines and (iii) not require knowledge of promoter and
modulatable transcriptional regulatory sequences.

In one example of the invention, mRNA is detected by
quantitative polymerase chain reaction.   Methods for
direct detection of mRNA in a rapid and quantitative
manner that fulfill the needs of a high throughput
screening method described in this invention are not
available. A suitable assay would have to meet the
following criteria: (i) specific detection of fewer than
50,000 mRNA molecules, (ii) short assay time of a few
hours, (iii) simple chemistry, that is amenable to
automation. Ideally a "single tube" assay would be used
which can be carried out within the same container in
which the cells to be tested have been cultured, by
eliminating transfer steps which may be time consuming
and contribute to assay variation.

Several techniques have been described by others, which
may be modified to be useful in the context of this
invention. All assays are based on the principle of using
a single stranded nucleotide probe containing sequences
complementary to the target mRNA transcribed from the

gene-of-interest which probe is attached to a solid
support to rapidly concentrate (capture) the mRNA out of
a crude cell lysate. Cell lysates can be prepared such
that ribonucleases are inhibited while hybridization of

5   nucleic acids remains possible, for example in high
concentrations of guanidinium isothiocyanate (47). Target
mRNA bound to the solid phase can be freed of
contaminants by intensive washing and subsequently
detected with a second probe which is complementary to a

10   different region of the target mRNA than the capture
probe. The second probe would carry an appropriate
detectable label.

To obtain optimal sensitivity i.e. obtain a maximal

15   number of labels being specifically bound to a target
molecule in the presence of a minimal number of labels
being unspecifically retained by other components of the
assay mixture, various techniques can be applied to
increase signal to noise ratios. A procedure termed

20   reversible target capture has been designed to reduce the
background noise by capturing the target molecule - label
complexes onto capture probes linked to solid phase,
release of the complexes and transfer of the complexes
onto new capture probes linked to solid phase and so

25   forth, cycling between steps. Background is reduced by
this procedure, because the release step is designed not
to release non-specifically bound label that will not
therefore be transferred together with the target
molecules and will eventually be removed from the mixture

30   (48). This method has a reported sensitivity of 15,000
target mRNA molecules, it has, however the disadvantage
of involving multiple transfer steps into new containers.

Beside reducing background the signal to noise ratio can

35   be improved by binding more than one label to a target

molecule or by creating many copies of an initial target
molecul (amplification). For example, DNA may be
detected by a method using an amplification
oligonucleotide with a branched structure that can
specifically bind to the target molecule and at the same
time to many molecules carrying label resulting in an up
to 500-fold amplification of specific signal. This method
can potentially be carried out in a single tube. Its
reported sensitivity is 60,000 target DNA molecules with
an assay duration of 4 hours (48). Further methods of
amplification include the hybridization dependant
replication of RNA via the Q-ß bacteriophage system (49)
or polymerase chain reaction based replication of a cDNA
copy of the target mRNA molecule (50). The methods
described herein above, have not been used by others in
the context of this invention. However, in light of the
subject invention, it would be clear to those skilled in
the art that use of these methods would facilitate the
practice of this invention.

The invention also provides the use of a reporter gene
whose product is easily detectable. The reporter gene
may encode a luciferase, chloramphenicol
acetyltransferase, ß glucuronidase, ß galactosidase,
neomycin phosphotransferase, or guanine xanthine
phosphoribosyltransferase.

The present invention also provides a screening method
for determining whether a molecule not previously known
to be a modulator of protein biosynthesis is capable of
transcriptionally modulating the expression of a gene-of-
interest which comprises separately contacting each of a
plurality of substantially identical molecules, e.g.
more than about 10^6 samples, preferably more than about

10^6 samples.

In theory, the ideal screening method provides contacting
one sample with a predetermined amount of each molecule
to be tested.   In practice, the method may include
contacting at least 3 substantially identical samples,
with a predetermined amount of each molecule to be
tested, more practically contacting about 9 substantially
identical samples with a predetermined amount of each
molecule to be tested.

Moreover, the invention provides a method of essentially
simultaneously screening molecules to determine whether
the molecules are capable of transcriptionally modulating
one or more genes of interest in a panel of such genes.
The   method   comprises   essentially   simultaneously
screening the molecules against each of the genes of
interest by separately contacting each of a plurality of
substantially identical samples, each of which contains
a predefined number of cells, with a predetermined amount
of a different molecule to be tested.

The screening method described hereinabove also provides
a quantitative testing range wherein at least about 10^3
samples per week are contacted with different molecules
against either one or a panel of such genes of interest.

Further, in the practice of this invention a method for
determining whether a molecule not previously known to be
a modulator of protein biosynthesis is capable of
transcriptionally modulating the expression of a gene-of-
interest is provided. This method works in human and in
animal cells. Data is presented so that one skilled in
the art could make the method work in fungal cells. In

51

addition, on skill d in th art would realize that the
m thod may be effected in plant cells or in bacterial
cells. In the case wherein luciferase is used as a
reporter, this enzyme has been shown by others to be
active in plant cells (89).

The invention also provides a method for
transcriptionally modulating in a multicellular organism,
the expression of a gene-of-interest, the expression of
which is associated with a defined physiological or
pathological effect in the organism. The method
comprises administering, e.g. oral administration,
administration as a suppository, topical contact,
intravenous, intramuscular or subcutaneous
administration, to the organism an amount of a molecule
effective to transcriptionally modulate expression of the
gene thereby affecting the defined physiological or
pathological effect. The molecule (a) does not naturally
occur in the organism, (b) specifically transcriptionally
modulates expression of the gene-of-interest, and (c)
binds to DNA or RNA or binds to a protein through a
domain of such protein which is not a ligand binding
domain of a receptor which naturally occurs in the
organism. Moreover, the binding of a ligand to the
ligand binding domain is normally associated with the
defined physiological or pathological effect.

In the practice of the invention, examples of a
multicellular organism include, but are not limited to,
a human, an animal, or a plant.

The defined pathological effect may be associated with a
disorder and the modulated expression of the gene-of-
interest may be associated with amelioration of the
disorder. Further, examples of disorders include but are

not limited to, a selection from the gr up consisting of
cancer, a hematopoietic dysfunction, diabetes, tissue
inflammation, atherosclerosis, dysfunctions of memory or
learning, dysfunctions in a cholesterol or other
metabolic pathway; viral, fungal or parasitic infections.
Thus, the gene-of-interest is not necessarily part of the
normal genetic make-up of the multicellular organism but
rather can be introduced via infection by a pathogen. In
one example of the invention, the defined physiological
effect is growth and the organism is an animal such as a
man, a cow, a pig, a bird, a fish, a sheep or a horse.
In another example of the invention the defined
physiological or pathological effect is an agronomically
important trait. In a further example of the invention
administration comprises topical contact. Further in
another example of the invention, administration
comprises oral, transdermal, intravenous, intramuscular
or subcutaneous administration. Further in one example
of the invention, provides the gene-of-interest encodes
a naturally occurring receptor. Further, in one example
of the invention, the receptor is a testosterone
receptor. Further, in another example of the invention
the receptor is an estrogen receptor. Further, in
another example of the invention, the receptor which
naturally occurs in the cell is a testosterone receptor.
Further, in another example of the invention the receptor
which naturally occurs in the cell is an estrogen
receptor. Further, in another example of the invention
the gene-of-interest encodes a TGF-ß receptor. Further,
in another example of the invention the TGF-ß is TGF-ß1.
Further, in another example of the invention the TGF-ß is
TGF-ß2. Further, in another example of the invention the
TGF-ß is TGF-ß3. Further, in another example of the
invention the gene-of-interest encodes an oncogene.
Further, in another example of the invention the oncogene

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is th _neu_ oncogene. Further, in another example of the invention the oncogene is selected from the group consisting of H-, N-, and K-_ras_ oncogenes.

5

Additionally, the method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest provides that growth may be the defined physiological effect and the organism is an animal such 10   as a cow, a pig, a bird, a fish, a sheep, or a horse.

Further, the method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest provides that the agronomically important trait 15   may be the defined physiological or pathological effect.

Additionally, the invention provides a method for enhancing the

expression of human growth hormone by a

cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:

effective to enhance the expression of human growth hormone by the cell.

Additionally, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:

effective to enhance expression of human growth hormone by, and thus growth of, the human being.

The present invention also provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:

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effective to enhance the expression of human growth hormone by the cell.

Another method of the invention provides increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:

effective to enhance expression of human growth hormone by, and thus growth of, the human being.

This invention also concerns a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:

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effective to enhance the expression of human growth
hormone by the cell.

Further, this invention provides a method of increasing
the growth of a human being ·who (i) comprises DNA
encoding, and (ii) is capable of expressing, human
growth hormone, comprising administering to the human
being an amount of a molecule having the structure:

effective to enhance expression of human growth hormone
by, and thus growth of, the human being.

Additionally, this invention concerns a method for
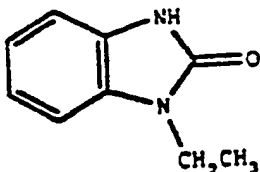enhancing the expression of human growth hormone by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, human growth hormone, comprising
contacting the cell with an amount of a molecule having
the structure:

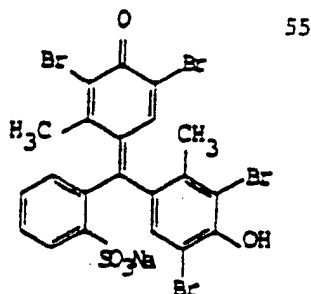effective to enhance the expression of human growth
hormone by the cell.

The pr sent invention further provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:

effective to enhance expression of human growth hormone by, and thus growth of, the human being.

The invention provides another method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:
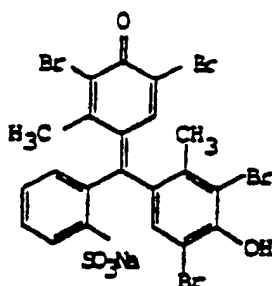
$$\text{wherein R is } \overset{\text{O}}{\underset{\text{O}}{\overset{\|}{\underset{\|}{\text{S}}}}}\text{-NHCH}_2\text{CH}_2\text{CH}_2\overset{\text{CH}_3}{\underset{\text{CH}_3}{\overset{|}{\underset{|}{\overset{+}{\text{N}}}}}}\text{CH}_3 \text{ or } -\ ^{-}\text{O}\overset{\text{O}}{\underset{\text{O}}{\overset{\|}{\underset{\|}{\text{S}}}}}\text{-OCH}_3,$$

effective to enhance the expression of human growth hormone by the cell.

Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:
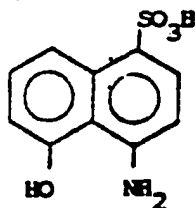

$$\text{wherein R is } \overset{\text{O}}{\underset{\text{O}}{\overset{\|}{\underset{\|}{\text{S}}}}}\text{-NHCH}_2\text{CH}_2\text{CH}_2\overset{\text{CH}_3}{\underset{\text{CH}_3}{\overset{|}{\underset{|}{\overset{+}{\text{N}}}}}}\text{CH}_3 \text{ or } -\ ^{-}\text{O}\overset{\text{O}}{\underset{\text{O}}{\overset{\|}{\underset{\|}{\text{S}}}}}\text{-OCH}_3,$$

effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Further, another method of the invention provides for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:
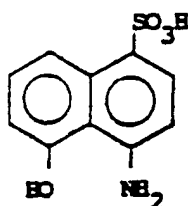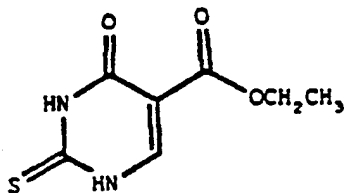
effective to enhance the expression of human growth hormone by the cell.

The invention provides yet another method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:
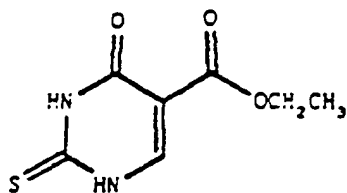
effective to enhance expression of human growth hormone
by, and thus growth of, the human being.

The present invention provides a method for enhancing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
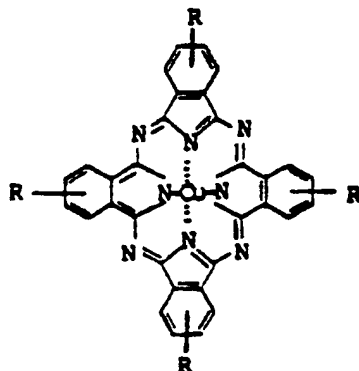of a molecule having the structure:

effective to enhance the expression of G-CSF by the
cell.

The present invention also provides a method for
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF. The method comprises
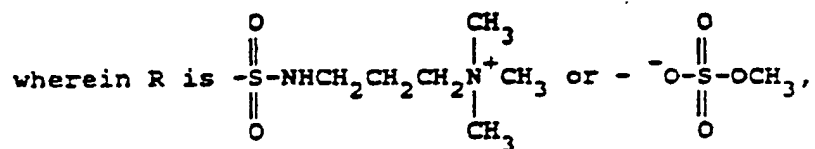administering to the human being an amount of a
molecule having the structure:

effective to enhance the expression of G-CSF by, and
thus increase the formation of neutrophils in, the
human being.

5   The present invention provides a method for enhancing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
of a molecule having the structure:

10

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effective to enhance the expression of G-CSF by the
cell.

Additionally, the invention provides a method of
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
20   capable of expressing, G-CSF. The method comprises
administering to the human being an amount of a
molecule having the structure:

25

eff ctive to enhanc expression of G-CSF by, and thus increase the formation of n utrophils in the human being.

A method of the invention involves enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
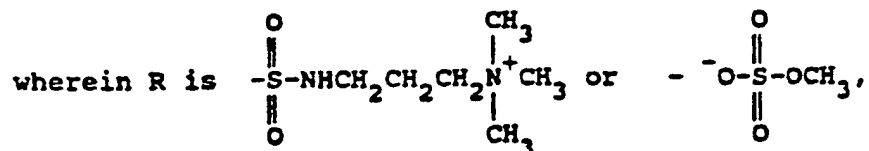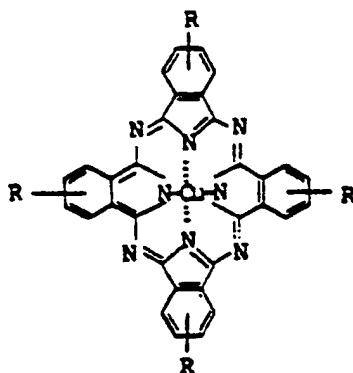
$$H_3C-N^+-CH_3$$
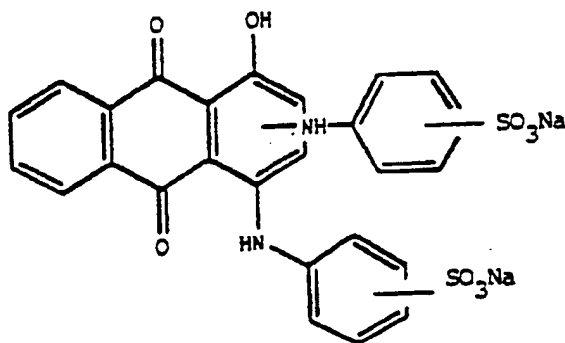
effective to enhance the expression of G-CSF by the cell.

Still another method of the invention involves increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:
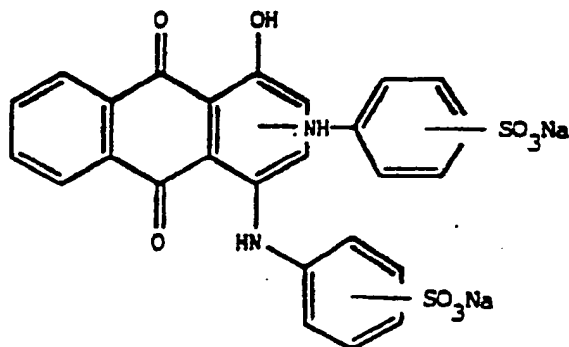
$$H_3C-N^+-CH_3$$

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eff ctive to enhanc expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

5   The present invention provides a method for enhancing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising contacting the cell with an amount of a
molecule having the structure:

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15   effective to enhance the expression of G-CSF by the
cell.
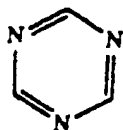
Additionally, the invention concerns a method of
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising administering
20   to the human being an amount of a molecule having the
structure:

25

64

effective to enhance expr ssion of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

This invention further concerns a method for enhancing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing G-CSF.
The method comprises contacting the cell with an amount
of a molecule having the structure:

$$
\begin{array}{ccc}
CH_3 & & CH_2CN \\
| & & | \\
N & & S \\
H_3C^{\diagup} & \diagdown C^{\diagup} & \\
& \| & \\
& S &
\end{array}
$$

effective to enhance the expression of G-CSF by the
cell.

Further provided is a method of increasing the
formation of neutrophils in a human being who (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF, comprising administering to the
human being an amount of a molecule having the
structure:

$$
\begin{array}{ccc}
CH_3 & & CH_2CN \\
| & & | \\
N & & S \\
H_3C^{\diagup} & \diagdown C^{\diagup} & \\
& \| & \\
& S &
\end{array}
$$

effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

Additionally, th present invention concerns a method
for enhancing the expression of G-CSF by a cell which
(i) comprises DNA encoding, and (ii) is capable of
expressing, G-CSF, comprising contacting the cell with
an amount of a molecule having the structure:

5

10
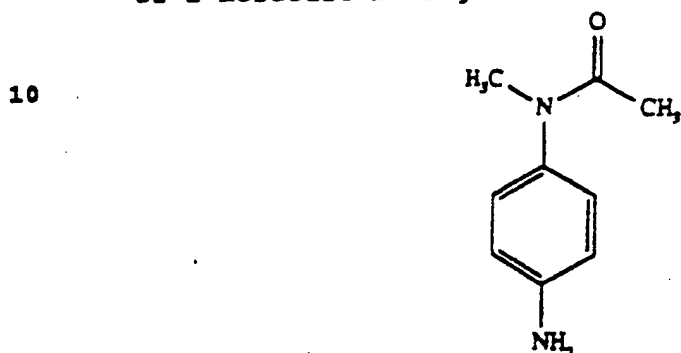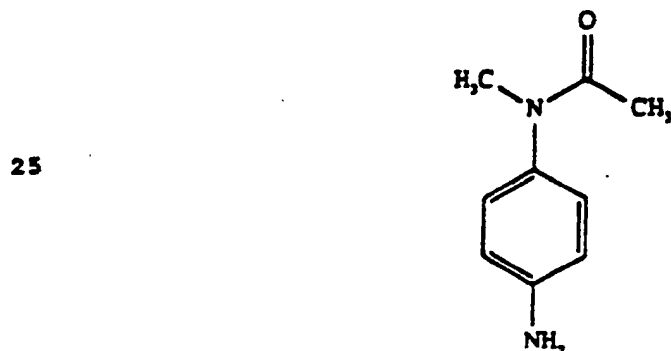
effective to enhance the expression of G-CSF by the
cell.

15

Also, the invention provides a method of increasing the
formation of neutrophils in a human being who (i)
comprises DNA encoding, and (ii) is capable of
expressing G-CSF, wherein the method comprises
administering to the human being an amount of a
molecule having the structure:

20

25

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Further, the invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
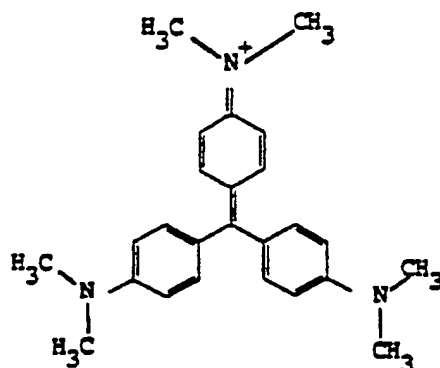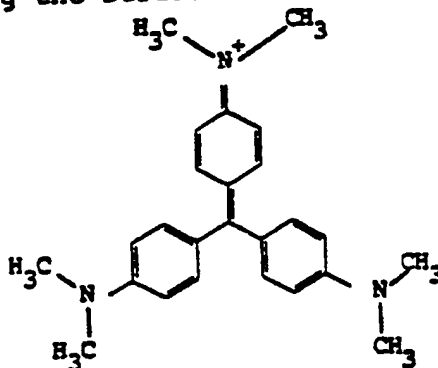
Br

Cl   Cl

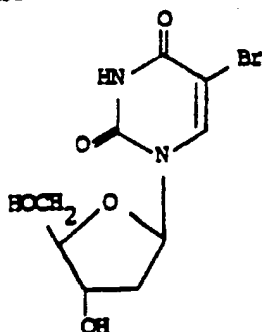effective to enhance the expression of G-CSF by the cell.

The invention additionally concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, wherein the method comprises administering to the human being an amount of a molecule having the structure:

Br

Cl   Cl

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

The invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

.HCl

.1/2 ZnCl2

effective to enhance the expression of G-CSF by the cell.

The invention further provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:

. HCl

. 1/2 ZnCl2

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in the human being.

Additionally, the invention provides a method for
enhancing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
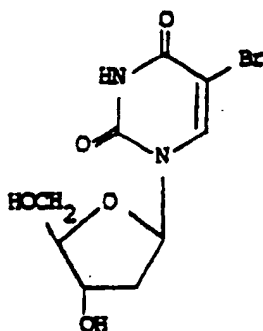cell with an amount of a molecule having the structure:

effective to enhance the expression of G-CSF by the
cell.

Further, the present invention provides a method of
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing G-CSF. The method comprises
administering to the human being an amount of a
molecule having the structure:

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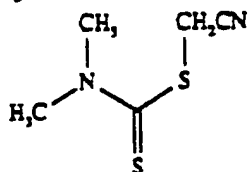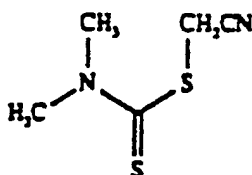H₃C and CH₃ structures with Cl⁻ counterions

effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

This invention also concerns a method of decreasing the
expression of G-CSF by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
of a molecule having the structure:

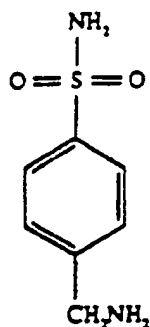effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, such as

superoxide or peroxide formation, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

Further, the invention also concerns a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
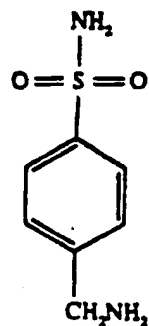cell with an amount of a molecule having the structure:

effective to d crease th   xpression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, such as
superoxide or peroxide formation, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:

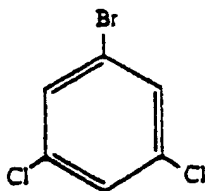effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

The invention further provides a method of decreasing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
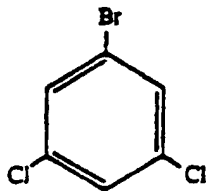of a molecule having the structure:

72

Br   CH₃

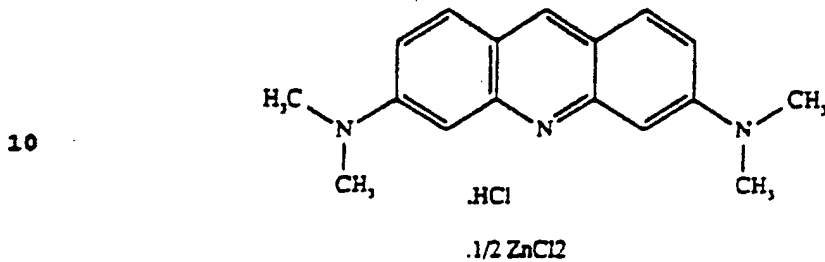effective to decrease the expression of G-CSF by the cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
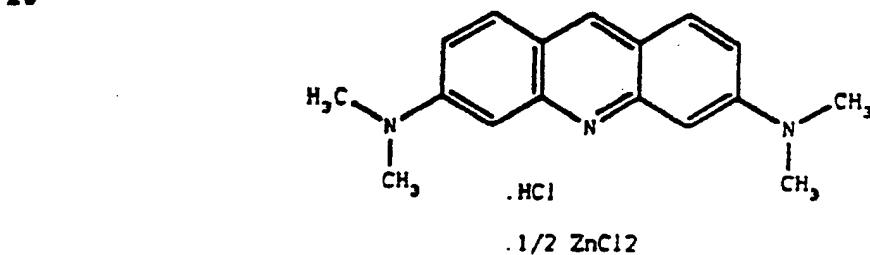
Br   CH₃

effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

Further, the present invention a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF.

The method comprises contacting the cell with an amount
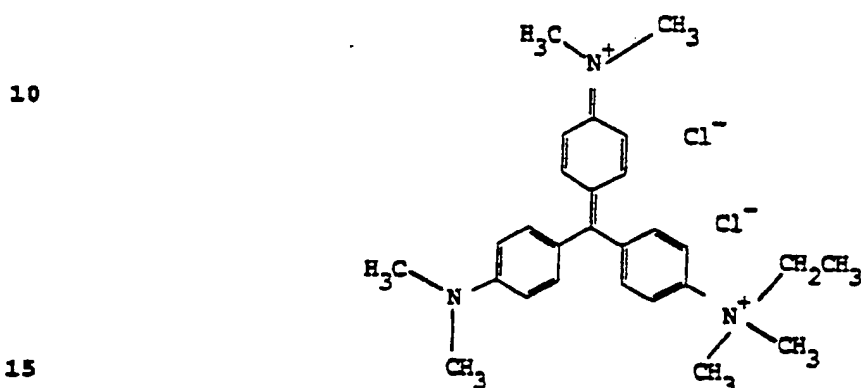of a molecule having the structure:

5

$$CH_2OH$$

(benzene ring with CH₂OH and Cl substituents)

10

effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
15   the metabolic functions of neutrophils, such as
superoxide or peroxide formation, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:

20

$$CH_2OH$$

(benzene ring with CH₂OH and Cl substituents)

25

effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

The present invention further provides a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
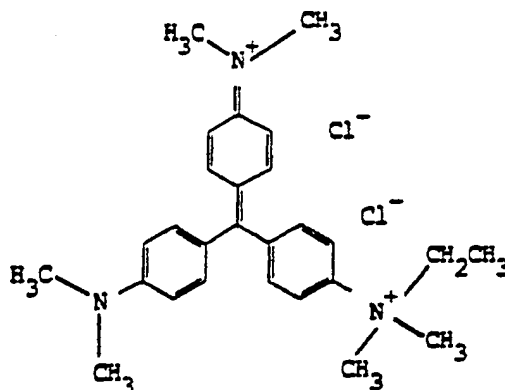

effective to decrease the expression of G-CSF by the cell.

Further still the invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
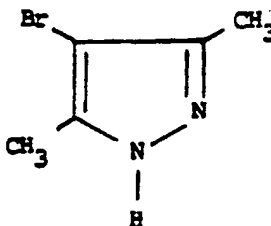
75

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

The present invention also concerns a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
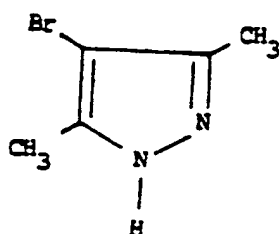cell with an amount of a molecule having the structure:

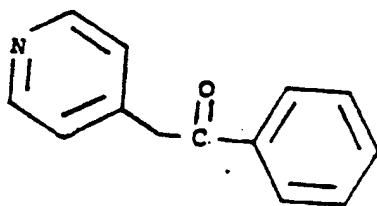effective to decrease the expression of G-CSF by the
cell.

Additionally, the present invention concerns a method
of decreasing the formation of neutrophils and
affecting the metabolic functions of neutrophils, such
as superoxide or peroxide formation, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,

comprising administering to the human being an amount
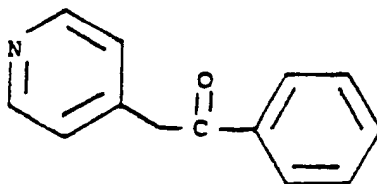of a molecule having the structure:

5

$CONH_2$

OH

10

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

15   The present invention also concerns a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
cell with an amount of a molecule having the structure:

20

OH   OH

HO   O
            C   OH

            OH

25

effective to decrease the expression of G-CSF by the
cell.

Further, the inv ntion concerns a method of decreasing the .formation of neutrophils and effecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
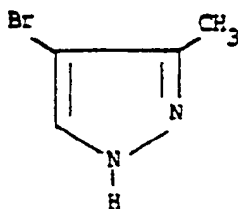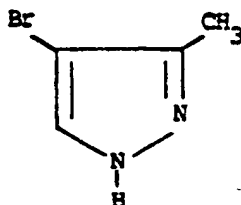

effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

Moreover, the invention provides a method of decreasing the expression of a mammary tumor virus, such as a mouse mammary tumor virus, by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, a mammary tumor virus. The method comprises contacting the cell with an amount of a molecule having the structure:

78
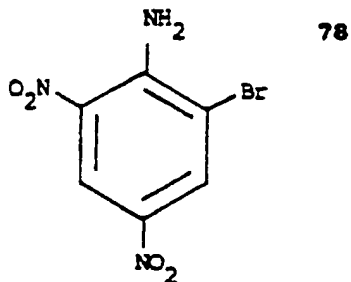
effective to decrease the expression of a mammary tumor
virus by the cell.

Finally, the invention also provides a method of
suppressing the proliferation of a mammary tumor virus
in a subject who (i) comprises DNA encoding, and (ii)
is capable of expressing, a mammary tumor virus,
comprising administering to the subject, e.g. a human
subject or an animal subject, an amount of a molecule
having the structure:
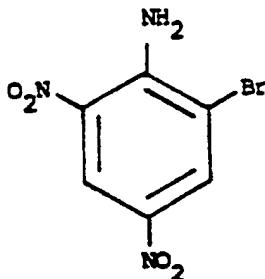
effective to suppress the proliferation of a mammary
tumor virus in the subject.

This invention is illustrated in the Experimental Details
and Results sections which follow. These sections are
set forth to aid in an understanding of the invention but
are not intended to, and should not be construed to,

limit in any way th invention as set forth in th claims
which follow.

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

5   A. Cell Culture

All media and reagents used for routine cell culture were
purchased from Gibco (Grand Island, NY), Hazelton
(Lenexa, KS), or Whittaker M.A. Biologicals
10   (Walkersville, MD). Fetal calf serum (FCS) was from
Hyclone (Logan, UT), and nutrients used for serum-free
defined media were purchased from Sigma (St. Louis, MO),
Boehringer Mannheim (Indianapolis, IN), Bachem (Torrance,
CA) and Collaborative Research (Bedford, MA).
15

NIH/3T3 fibroblast cells (ATCC number CRL 1658) were used
for transfection of plasmids containing the mouse mammary
tumor virus (MMTV) promoter linked to firefly luciferase
coding sequences (see below). Cells were propagated in
20   Dulbecco's modified Eagle's medium (DMEM) obtained from
Gibco, Grand Island, NY and supplemented with 10% FCS.
For high-throughput (HTP) screening, transfected NIH/3T3
clones were transferred to serum free defined medium
consisting of Iscove's modified Eagle's medium (IMEM) and
25   Ham's F12 medium (1:1) supplemented with growth factors,
hormones and nutrients as described previously (43).

A rat pituitary cell line, designated GC, (4, 25) was
used for transfection of plasmids containing the human
30   growth hormone promoter (see below) and was maintained in
DMEM and Ham's F12 medium (1:1), supplemented with 12.5%
FCS. For HTP screening, transfected GC clones were
transferred to serum free defined medium consisting of
DMEM and Ham's F12 medium (1:1) supplemented with growth
35   factors, hormones and nutrients as described previously

81

(17, 5).

A human bladder carcinoma cell line (U5637, ATCC number
HTB 9) was used for transfection of plasmids containing
5   the human Granulocyte-Colony Stimulating Factor (G-CSF)
promoter (see below) and was maintained in RPMI medium
supplemented with 10% FCS.   For HTP screening,
transfected 5637 clones were transferred to a serum free
defined medium identical to that used for the NIH/3T3
10   clones.

G418 (Geneticin, Gibco) at 0.2 mg/ml was routinely added
to both serum and serum free defined media for
maintenance of cell lines transfected with the neomycin
15   resistance gene.

B.   Plasmid Construction and Molecular Cloning of
Promoter-Reporter Fusion Constructs to be
Transfected into Cells Used for a 2,000-Chemical
20   Transcription Screen

This section describes (a) the molecular cloning of the
human G-CSF promoter and adjacent 5' transcriptionally
modulatable regulatory sequences and (b) the making of
25   constructs where these regulatory sequences or those of
the human growth hormone (hGH) gene or those of the mouse
mammary tumor virus (MMTV) long terminal repeat (LTR)
control the expression of the firefly luciferase gene.
These constructs were transfected into cells as described
30   in Section C and used for a high-throughput pilot screen
of 2,000 chemicals to identify chemicals acting as
specific transcriptional modulators (see Section E and
"Results").

35   Unless otherwise indicated cloning procedures were

82

performed essentially according to Maniatis et al. (1982)
(28). Oligonucleotides were synthesized by the
beta-cyanoethyl phosphoramidite method according to
protocols provided by the manufacturer of the
5   DNA-synthesizer (Model 380A, Applied Biosystems (Foster
City, CA).

1. Construction of the MMTV Promoter-Luciferase fusion
plasmid (pMluci)

10

The firefly luciferase gene was removed from the plant
expression plasmid pDO432 (33) (Figure 1) as a 1.9 kb
BamHI fragment and cloned into the BamHI site of pSVL
(Pharmacia, Piscataway, NJ), a mammalian expression
15   vector containing the SV40 promoter. The resulting
plasmid (pSVLuci; Figure 2) was digested with XhoI and
SalI to produce a 2.4 kb fragment containing the
luciferase coding sequences and the SV40 late
polyadenylation site. This fragment was inserted into
20   the XhoI site of pMSG (Pharmacia, Piscataway, NJ), a
eucaryotic expression vector containing the MMTV
promoter. The resulting MMTV promoter-luciferase fusion
plasmid (pMLuci; Figure 3) was used to transfect NIH/3T3
cells as described below (section C1). Similar
25   constructs can be made using luciferase vectors from
Clontech (Palo Alto, CA).

2. Construction of the human growth hormone (hGH)
promoter-luciferase fusion plasmid

30

The SalI-XhoI fragment of pSVLuci (Figure 2) containing
the luciferase coding sequences and the SV40 late
polyadenylation site was inserted into pUC 8 (Biorad,
Richmond, CA), which had been linearized by a SmaI/HincII
35   digestion and ligated to XhoI linkers (New England

83

Biolabs, Beverly, MA). The n w plasmid thus generated
(pUXLuci; Figur 4) was linearized by XhoI digestion
followed by incubation with the Klenow fragment of E.
coli DNA polymerase and the four deoxyribonucleotides to
5   fill in the single-stranded ends of the vector. This
linear (5.1 KB) form of pUXLuci was then ligated to the
filled-in 550bP HindIII-XbaI fragment of the plasmid
phGH:CAT (Figure 5) (25). Human growth hormone promoter
sequences located on the HindIII-XbaI fragment were thus
10   fused to the luciferase coding sequences located on
pUXLuci generating the plasmid phGH-Luci (Figure 6),
which was used in transfections of GC cells as described
below (Section C2).

15   3.   Construction   of   the   human   Granulocyte-Colony
stimulating factor (hG-CSF) Promoter-Luciferase fusion
plasmid (pG-Lucl)

Information on the G-CSF upstream and coding sequences
20   was published by Nagata et al. (1986) and was used to
synthesize 5 oligonucleotide probes (OL-1 to OL-5) to
screen a human leukocyte genomic DNA library (Clontech,
Palo Alto, CA) according to the supplier's instructions.
The sequences of the oligonucleotide probes were:

25

5' GCTTTTTGTTCCAACCCCCCTGCATT 3' (OL-1);
5' CCCTGCATTGTCTTGGACACCAAAT 3' (OL-2);
5' GCGCTCCAGGAGAAGCTGGTGAGT 3' (OL-3);
5' AAGCTGATGGGTGAGTGTCTTGGC 3' (OL-4);
30   5' ATCAGCGGCTCAGCCTTCTT 3' (OL-5);

The sequences of OL-1, OL-2 and OL-5 recognize the G-CSF
promoter region, OL-4 recognizes the first intron/exon
junction and OL-3 recognizes sequences within the second
35   exon (32). One of the clones isolated from the leukocyte

84

library using these oligonucle tide probes contains a 3.5
kb SalI-BamHI fragment of G-CSF genomic sequence
consisting of 3.3 kb of promoter sequence and two hundred
base pairs of the coding region. This fragment was
inserted into the vector pGEM-7-Zf (Promega, Madison, WI)
which had previously been digested with SalI/ BamHI,
resulting in the vector pJM710 (Figure 7). pJM710 was
then digested with PstI, and the resulting 1.6 kb
fragment containing G-CSF upstream sequences and the
first 15 bases of the G-CSF leader sequence was inserted
into the PstI site of pGEM5-Luci (Figure 8) to generate
pG-Lucl (Figure 9). This construct was then used for
transfections of 5637 human bladder carcinoma cells as
described below in section C3. pGEM5-Luci (Figure 8) had
previously been constructed by inserting the XbaI/SalI
fragment from pSVLuci (Figure 2) containing the
luciferase coding sequence and the SV40 late
polyadenylation signal into pGEM 5-Zf (Promega, Madison
Wl) digested with XhoI/SalI.

C. Construction of Single Cell Clones Containing Various Promoter-Luciferase Fusion Constructs

1. pMluci

pMluci (Figure 3) and pSV2Neo, an antibiotic resistance
plasmid (34), were co-transfected into NIH/3T3 mouse
fibroblast cells using the calcium phosphate
precipitation method (15) with a commercially available
kit (Pharmacia, Piscataway NJ). Two days later, cells
were transferred to media containing 0.4 mg/ml G418 and
were grown for an additional 10-14 days. G418-resistant
clones were isolated by standard methods. Once
sufficient cell numbers were obtained, clones were
analyzed based on several criteria: constitutive

luciferase production, induction of luciferase expression
by dexam thas ne (1 μM Sigma, St. Louis, MO),
satisfactory attachment to microtiter plates used in the
high-throughput screen (see section E) and acceptable
standard deviation in multiple luciferase expression
assays (see below for assay protocol). This analysis was
carried out using the luciferase assay conditions
described in sections D and E provided herein. Of the
clones which satisfied the above criteria for the high
throughput screen, one clone, M10, was selected for use.

2. phGH-LUCI

phGH-LUCI (Figure 6) and pRSVNeo, an antibiotic
resistance plasmid (14), were co-transfected into GC rat
pituitary cells as described above. Selection of
G418-resistant cell clones was described above except for
using a concentration of 0.2 mg/ml G418. Analysis of the
cell clones was performed as above, except that known
inducers of hGH expression (10-100 nM rat growth hormone
releasing factor (rGRF, Bachem, Torrance, CA) and 10μM
forskolin (Sigma, St. Louis, MO) were used in place of
dexamethasone. One clone, 532, was selected for further
use in the high throughput screen.

3. pG-LUC1

pG-LUC1 (Figure 9) and pRSVNeo were co-transfected into
5637 human bladder carcinoma cells as described above.
Selection of G418 resistant clones was as described above
except for using a concentration of 0.1 mg/ml G418.
Analysis of cell clones was performed as above except
that a known inducer of G-CSF expression (1-5 μg/ml
lipopolysaccharide (LPS), E. coli serotype 055:b5, Difco,

Detroit, MI or Sigma, St. Louis, MO) was used in place of dexamethason . One clone, G21, was selected for use.

D. Liquid Scintillation Counter Bioluminescence Assay

To assay for luciferase expression in transient expression assays in the various transfected clones, cells were incubated with various transcriptional inducers in serum free defined media, washed 3 times with Dulbecco's phosphate-buffered saline (D-PBS, Gibco) and lysed and measured according to the method of DeWet, et al. (100). Results were normalized to protein concentration using the Bradford protein assay (BioRad, Richmond CA) or to cell numbers using Trypan Blue (Sigma) exclusion counting in a hemocytometer (see section E).

E. High-Throughput (HTP) Screening

Cell plating: 96 well plates were treated with 50 μl per well of human fibronectin (hFN, 15 μg/ml in PBS, Collaborative Research, Bedford, MA) overnight at 37C. hFN-treated plates were washed with PBS to remove excess hFN prior to cell plating. M10, 532, and G21 cells maintained in their respective serum media (with 0.2 mg/ml G418) were washed with PBS, harvested by trypsinization, and counted using a hemocytometer and the Trypan Blue exclusion method according to protocols provided by Sigma, St. Louis, MO Chemical Company. Cells were then diluted into serum free defined media (with 0.2 mg/ml G418), and 0.2 ml of cell suspension per well was plated onto microtiter plates (532 and G21) or hFN-treated plates (M10). Plates were incubated overnight at 37C in a humidified 5% CO_2 atmosphere.

Addition of Chemicals to Cells: Chemicals from the

Oncogene Science file were dissolved in DMSO at
concentrations of 3-30 mg/ml and diluted 5 fold, 110
fold, and 726 fold. 10 μl of each dilution were added to
each of quadruplicate samples of cells contained in the
5   wells of 96-well microtiter plates. Cell plates were
then shaken and incubated for 6 hours at 37C, 5% CO_2.

Bioluminescence Assay: After incubation with OSI-file
chemicals, cell plates were washed 3 times with PBS.
10   Cells were lysed and bioluminescence was measured in a 96
well luminometer according to the method of DeWet, et al.
(100). Data were captured using Lotus-Measure (Lotus)
software and processed by custom-designed macros written
in Lotus.

15

·F. **Isolation of Total Cellular RNA**

Total cellular RNA was isolated from the G21 cell clone
20   or from untransfected 5637 cells following incubation for
6 hours with various transcriptionally modulating
chemicals identified in the high-throughput screen.
Cells were grown in serum free medium as described above.
Total cellular RNA was isolated using the RNAZol method
25   (CINNA/BIOTECX, Friendswood, TX, Laboratories
International, Inc.). Cells were resuspended and lysed
with RNAZol solution (1.5 ml/9 cm petri dish) and the RNA
was solubilized by passing the lysate a few times through
a pipette. Chloroform was added to the homogenate (0.1
30   ml/1 ml), and samples were shaken for 15 seconds followed
by a 5 minute incubation on ice. After centrifuging for
10 minutes, the upper phase was collected and an equal
volume of isopropanol was added. Samples were incubated
for 45 minutes at -20C, and the RNA was pelleted for 15
35   minutes at 12,000 x g at 4C. The RNA pellet was then

washed with 70% ethanol and dried briefly under vacuum.

G. Northern Blotting

5   Total cellular RNA was isolated from 5637 cells following
       incubation with chemicals as described above and
       electrophoresed in a 1% Agarose-Formaldehyde gel. The
       RNA was transferred to Duralon-UV nylon filters
       (Stratagene, La Jolla, CA) using the manufacturer's
10   recommended protocol. The filters were prehybridized for
       4 hours (prehybridizing solution = 5X SSC, 50 mM sodium
       pyrophosphate, 10X Denhardt's solution, 10% dextran
       sulfate, 7% SDS and 250 lg/ml denatured ssDNA) and then
       hybridized in the same solution for 16 hours at 65C in
15   the presence of G-CSF or Beta-Actin (Oncor, Gaithersburg,
       MD) specific probes. The G-CSF probe was a 0.6kb AflII
       to Xhol fragment which contained most of exon 5 of the
       human G-CSF gene. The B-actin probe was used as a
       control probe to normalize for the total amount of RNA.
20   The probes were labeled with alpha-^{32}P dCTP using a
       random primed DNA labeling kit (Amersham, Arlington, IL).
       Following hybridization, filters were first probed with,
       G-CSF-and Fusion reprobed with B-Actin Probe were washed
       three times at room temperature with 1X SSC, 0.13% SDS
25   and three times at 65C with 0.2X SSC, 0.1% SDS. Filters
       were first probed with G-CSF-and then reprobed with
       B-actin-probe. Exposure to x-ray films was performed
       overnight. Bands were excised and counted in a liquid
       scintillation counter (LKB, Gaithersburg, MD), and counts
30   obtained with the G-CSF specific probe were normalized
       relative to the counts obtained with the B-Actin specific
       probe.

35   H. S1 Nuclease protection

89

S1 Nuclease protection assays were carried out as described in reference 94.

5   I. MTT Cell toxicity assay

To determine cytotoxic concentrations of chemicals registering as positives in the High-Throughput luciferase assay the MTT cytotoxicity assay was employed
10   (95). In this assay, a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, MTT] is reduced to a colored formazan product by reducing enzymes present only in living metabolically active cells.

15

J. Two-antibody Sandwich Immunoassay

Supernatants from 5637 bladder carcinoma cells incubated with chemicals registering as positives in the G-CSF
20   promoter/luciferase High-Throughput assay were assayed for secreted G-CSF protein using the two-antibody sandwich immunoassay (96). The G-CSF Assay kit manufactured by Oncogene Science, Inc. was used and the manufacturer's instructions were followed.

25

K. Construction of a Yeast Expression Vector

Plasmid pHZ18 (97,98) contains 2µ DNA for propagation in S. cerevisiae, the yeast promoter cyc 1, which is
30   compatible with expression in yeast cells, and the URA 3 gene for selection. The plasmid was linearized with BamH1, the ends were filled-in using deoxynucleotides and E.coli DNA polymerase Klenow fragment, and then the plasmid was digested with Aat II. A 4.1 kb fragment
35   containing the cyc1 promoter, URA3 and 2µ genes was

separated by agar se gel electrophoresis and subsequently purified by electroelution onto ion-exchange paper (Whatman, DE81). Plasmid pBR322 was treated with endonucleases Aat II and Pvu II and a 2.2 kb fragment
5   containing the plasmid's origin of replication and the amp^R gene was isolated by agarose gel electrophoresis and eluted onto DE81 paper.

The 2.2 kb pBR322 and 4.1 kb pHZ18 fragments were ligated
10   using T4 DNA ligase according to standard procedures (94). The resulting 6.3 kb vector pHZBR was digested with BamH1 for subsequent insertion of the luciferase coding sequence downstream of the cyc1 promoter.

15   An Nco I – Sal I restriction fragment containing the luciferase gene starting at the second ATG, was made blunt-ended by filling in and ligated into the filled-in BamH1 site of pHZBR as readily understood by one skilled in the art. Clones of the correct orientation were
20   identified via restriction mapping to yield plasmid pHZluci24. This plasmid was used to transform <u>S.cerevisiae</u> strain DB745.

<u>L. Transformation of Yeast Cells</u>
25

<u>S.cerevisiae</u> DB745 were made competent according to published methods (99). One and 4μg of either pHZluci24 or pHZ18 (transfection control) were added to the competent cells and incubated at 30°C for 30 minutes.
30   Lithium acetate-PEG was mixed gently with the cells and allowed to incubate for another 45 minutes at which time the cells were shifted to 42°C for 5 minutes. The cells were spread onto uracil(-) plates and incubated at 30°C for several days. Cell colonies were picked, grown to
35   saturation in YPD media and analyzed for luciferase

91

activity (99) Cell lysis and analysis of luciferase
activity can be carried out by methods known to one
skilled in the art (99, 100). Stock cultures were made
from clones expressing luciferase positive clones.

5

RESULTS

A. Validation of Cell Lines

10   Prior to initiation of drug screening, it was
demonstrated that the transfected promoter-luciferase
fusion plasmids were reacting to transcriptional inducers
in a manner as predicted based on the published
literature. As shown in Figure 10, all three transfected
15   cell clones chosen responded to inducers which have been
reported to stimulate the endogenous genes; the
·MMTV-luciferase containing clone M10 was stimulated 11.6
fold by 1μM dexamethasone, the hGH-luciferase containing
clone 532, was stimulated 2.2 fold by 100 nM rat growth
20   hormone releasing factor (GRF), and the hG-CSF containing
clone G21 was stimulated 5.7 fold by 5 μg/ml
lipopolysaccharide (LPS).

25   It was also demonstrated that certain steroidal chemicals
other than dexamethasone modulated luciferase expression
in the cell clone M10, which harbors the MMTV
promoter-luciferase fusion construct. As shown in Figure
11, dexamethasone stimulated the MMTV promoter in cell
30   clone M10 (mouse fibroblast origin), while progesterone
did not. It has been shown that a rat fibroblast cell
line which contains high levels of glucocorticoid
receptor but low levels of progesterone receptor, shows
stimulation of the MMTV promoter by the glucocorticoid
35   dexamethasone but not by progesterone (7). In addition,

92

Figure 11 shows that the mineralocorticoid aldoster ne
stimulates clone M10, as is expected based on previously
published work which indicates that aldosterone can act
through the glucocorticoid receptor to stimulate the MMTV
5   promoter (6).

A 7-hour incubation of G 21 cells with PMA in serum-free
media increased luciferase expression directed by the G-
CSF promoter by 34.6 fold in the absence and by 24.6 fold
10   in the presence of EGF. TNF-Alpha induction did not
significantly change on EGF addition (2.8 fold with and
2.1 fold without EGF).

B. High-Throughput Drug Screen
15

Table 1 shows a summary of the results of a one-week,
high-throughput screen of 2,000 chemicals to identify
those chemicals specifically stimulating or inhibiting
transcription from the G-CSF, hGH or MMTV promoters.
20   This screen concurrently tested chemicals at three
concentrations on quadruplicate samples of the M10, 532
and G21 cell lines. A minimum stimulation of one
promoter, to the degree indicated, and less than 50%
activation of the other two promoters was required for

93

TABLE 1

5

SUMMARY OF HIGH-THROUGHPUT SCREEN

10

Number (%) of Chemicals Which Activate Expression:

Promoter	2-3 Fold	3-5 Fold	5-7 Fold	7-10 Fold	>10 Fold	Total
G-CSF	NA	23 (1.1%)	10 (0.5%)	3 (0.15%)	2 (0.10%)	38 (1.9%)
hGH	NA	NA	12 (0.6%)	5 (0.03%)	6 (0.03%)	23 (1.14%)
MMTV	15 (0.7%)	1 (0.05%)	0 (0%)	1 (0.05%)	1 (0.05%)	18 (0.9%)

25

Number (%) of Chemicals Which Inhibit Expression >3 Fold

<u>Promoter</u>

30

G-CSF	7	(0.35%)
hGH	42	(2.1 %)
MMTV	1	(0.05%)

35

TABLE 2

A) TRANSCRIPTIONAL ACTIVATORS

		FOLD INDUCTION RELATIVE TO SOLVENT CONTROL		
Chemical #	Chemical Name	GCSF	hGH	MMTV
G-CSF:				
40	3-Acetyl-2-6-Bis(tertiary butyl amino)-4-methyl-pyridine	5.62	0.62	0.27
58	1-Acetylimidazole	6.03	0.17	0.42
237	N-Carbethoxy-phthalimide	4.77	0.06	0.62
254	1-(2-Chloroethyl)piperidine	4.09	0.90	0.98
364	Melamine	3.67	1.18	1.07
473	1,3,5,-Triazine	>3	0.50	0.87
542	5-Bromo-2'-deoxycytidine	6.28	1.08	1.26
543	5-Bromo-2'-deoxyuridine	7.17	0.72	0.98
878	Blueberry leaf extract	3.84	1.17	0.78
1025	Culvers Root extract	4.09	0.98	1.24
1234	4-Aminocinnamic Acid hydrochloride	4.97	0.51	1.03
1255	1-Bromo-3,5-dichlorobenzene	6.74	0.43	1.09
1374	4'-Amino-N-methylacetanilide	11.03	0.05	1.05
1375	4'-(aminomethyl)benzene sulfonamide hydrochloride	8.94	0.04	1.37
1376	2-Amino-5-Methyl benzene sulfonic acid	6.37	0.04	1.32
1397	5-Amino-3-methyl isothiazole hydrochloride	3.63	0.57	1.13
1482	2-Aminophenyl disulfide	3.99	0.54	1.07
1483	4-Aminophenyl disulfide	4.64	0.38	1.09

TABLE 2 (CONT.)

FOLD INDUCTION

Chemical#	Chemical Name	GCSF	hGH	MMTV
1521	2-Amino-6-purinethiol	3.59	0.73	0.92
1583	8-Bromoadenosine	5.82	0.12	0.88
1592	Bis(2,2,3,3,4,4,5,5,6,6,7,7,) dodecafluoroheptyl-(+)-camphorate	3.20	0.74	1.34
1783	Cupferron	6.55	0.32	0.89
1793	Cyanomethyl-N,N-dimethyl dithiocarbamate	9.50	0.52	1.21
1994	3-Bromobiphenyl	3.29	0.34	0.63
2001	1-Bromo-4-tertiary butyl benzene	3.11	0.74	1.12
2030	4-Bromo-2-fluoro-6-nitroanizol	5.53	0.57	0.87
2096	(+)-1-Bromo-3-Chloro-2methyl propane	3.27	0.61	0.89
2097	1-Bromo-5-Chloro pentane	5.09	0.88	1.22
2129	4-Chlorobenzyl Chloride	3.23	0.75	0.95

GROUP A:

378	7-Oxo-7H-benzo[e]perimidine 4-carboxylic acid	4.12	0.26	0.59
423	Quinacrine dihydrochloride hydrate	2.39	0.56	0.64
427	Resazurin	3.14	0.43	0.71
836	Thionin	3.20	0.23	0.58
1776	Cresyl Violet Acetate	3.50	0.15	1.36
1904	9-Aminoacridine hydrochloride	4.12	0.54	0.82

GROUP B:

670	Methyl Green	>3	0.52	0.79
1780	Crystal Violet	20.39	0.38	1.15

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TABLE 2 (CONT.)

FOLD INDUCTION

Chemical#	Chemical Name	GCSF	hGH	MMTV
GROUP A AND B:				
80 hGH:	Acridine Orange	5.87	0.65	0.63
70	2-Acetylpyrrole	0.43	9.26	0.85
299	10,11-Dihydrocarbamazepine	0.53	5.46	0.47
322	1-ethyl-2-benzimidazolinone	0.60	11.18	1.12
325	Fisetin	0.14	5.42	1.0
552	3-(4-chlorophenyl)-1-methoxy-1-methyl urea	0.81	5.31	0.86
790	Rivanol	0.01	5.94	0.58
792	Rose Bengal	0.94	5.31	1.21
856	Tripalmitin	0.28	6.49	0.42
1004	Arnica 4x	0.85	6.48	1.22
1160	Rochester # 6180	0.38	5.79	0.80
1251	Bromocresol Green	0.14	15.19	0.33
1337	4-Amino-5-hydroxy-1-naphthalene sulfonic acid	0.07	15.87	0.23
1499	2-Amino-4-phenylthiazole hydrobromide monohydrate	0.24	5.55	0.61
1550	2-Aminothiazole	0.04	5.44	0.87
1552	2-amino-2-thiazoline	1.23	7.26	0.52
1561	4-Amino-3,5,6-trichloropicolinic acid	0.23	8.05	0.48
1598	N,N'-Bis-[3-(4,5-dihydro-1H-imidizol-2-yl)phenyl] urea dipropanoate	0.72	5.32	1.27

97

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1678	4,8-Bis(hydroxymethyl)-tricyclo [5,2,1,0$^{2.6}$]decane	0.36	7.08	0.89
1740	5-carbethoxy-2-thiouracil	0.74	17.77	0.87
1747	N$_6$-carbobenzyloxy-L-lysine	0.78	6.16	0.88
1804	Cyclobutane carboxylic acid	1.05	9.41	0.49
1876	Alec Blue	0.87	11.91	0.40
1881	Alizarin Blue Black B	0.21	18.87	0.89

MMTV:

189	Bathocuproinedisulfonic Acid disodium salt hydrate	1.06	1.47	2.80
453	2,2':6',2"-Terpyridine	0.79	0.58	13.30
519	b-Apo-8'-carotenal	1.15	0.68	2.76
562	Copaiva Balsam	1.10	0.15	2.34
829	Homoveratric acid	0.85	1.05	2.48
833	5-Iodorotic acid	1.02	0.86	2.46
765	Prednisolone-21-Acetate	0.96	1.30	2.68
828	2,4,5,4'-Tetrachlorodiphenylsulfide	1.47	1.34	2.20
848	Triamcinolone acetonide	0.75	1.28	2.43
944	Peanut	1.15	0.91	2.10
1269	5-Amino-4,6-dichloropyrimidine	0.72	0.91	2.18
1316	2-Aminofluorene	0.74	1.39	2.33
1318	2-Amino-9-fluorenone	1.13	0.85	2.41
1384	2-Amino-4'-methylbenzophenone	1.33	0.50	2.43

98

TABLE 2 (CONT.)

			FOLD INDUCTION		
Chemical#	Chemical Name		GCSF	hGH	MMTV
1573	5-Bromoacenapthene		1.49	0.34	4.30
2064	4-(Bromomethyl)-6,7-dimethoxy-coumarin		0.82	1.10	2.53
2148	2-chlorocyclohexanone		0.45	0.92	2.82
2191	Chloramphenicol		0.37	0.35	7.32

B) TRANSCRIPTIONAL INHIBITORS

		FOLD INHIBITION RELATIVE TO SOLVENT CONTROL		
Chemical#	Chemical Name	GCSF	hGH	MMTV
G-CSF:				
209	4-Benzoylpyridine	6.66	1.08	0.81
371	Morin hydrate	11.11	0.41	0.89
660	Maclurin	10.0	0.34	1.04
798	Salicylamide	4.76	0.90	0.68
2009	4-Bromo-3,5-dimethylpyrazole	3.70	0.57	0.64
2082	4-Bromo-3-Methylpyrazole	5.26	0.65	1.23
2121	3-Chlorobenzyl alcohol	4.76	0.40	1.14
hGH:				
183	Auramine O	0.72	4.00	0.70
240	Carminic acid	0.63	5.26	0.80
443	Sulfamethazine	0.60	4.76	0.79
512	Amaranth	0.81	5.26	0.68

99

TABLE 2 (CONT.)

			FOLD INHIBITION		
	Chemical#	Chemical Name	GCSF	hGH	HMTV
5	541	5-Bromo-4-Chloro-3-indoxyl-phosphate K-salt	0.90	6.25	0.86
10	556	Chromazurol S	0.73	33.33	0.87
	561	Clove Oil	0.62	5.00	0.05
15	577	Na-Ne-Diacetyl-L-lysine	0.84	4.00	0.88
	578	Dibenzoyl-D-tartaric acid	0.85	4.00	0.91
20	630	Hydantoin-5-acetic acid	0.70	3.57	0.74
	840	Kernechtrot	0.84	5.00	0.59
	759	Piperidine	0.84	5.88	0.95
25	764	Prednisolone	0.82	4.54	0.59
	875	Black Walnut extract	0.69	6.25	0.80
30	892	Colts Foot Leaves extract	0.88	11.11	0.87
	893	Comfrey Leaf extract	0.74	11.11	0.90
	920	Horehound Herb extract	0.56	3.84	0.84
35	921	Horsetail Grass extract	0.72	3.44	0.86
	942	Pau D'Arco extract	0.80	6.25	0.63
40	970	Thyme extract	0.57	4.34	1.07
	1591	1,2-Bis(di-p-tolylphosphino)-ethane	0.56	5.55	0.96
45	1604	2,4-Bis[5,6-bis(4-sulfophenyl)-1,2,4-Triazine-3-yl)-pyridine, tetrasodium salt hydrate	0.77	5.00	0.97
	1635	[(1S)-endo]-(-)-Borneol	0.71	9.09	0.99
50	1640	1,2-Bis(2-pyridyl)-ethylene	0.79	5.00	0.59
	1641	2,3-Bis(2-pyridyl)-pyrazine	0.83	5.55	0.60

100

TABLE 2 (CONT.)

			FOLD INHIBITION		
	Chemical#	Chemical Name	GCSF	hGH	MMTV
	1648	2-[5,6-Bis(4-sulfophenyl)-1,2,4-triazine-3-yl]-4-(4-sulfophenyl)-pyridine, trisodium salt	0.86	7.69	1.00
	1651	Bis(2,2,2-trifluoroethyl) (methocarbonyl-methyl)-phosphonate	0.69	3.57	0.70
	1655	2,5-Bis(trifluoro-methyl)benzoic acid	0.54	4.76	0.81
	1703	3-Bromobenzonitrile	0.76	10.00	0.90
	1704	4-Bromobenzonitrile	0.77	4.16	0.94
	1705	4-Bromobenzophenone	0.54	14.28	0.62
	1712	Calcein Blue	0.74	8.33	0.94
	1720	(1S)-(-)-Camphor	0.65	4.76	0.66
	1764	7-(Carboxymethoxy)-4-Methylcoumarin	0.55	7.14	0.82
	1770	Carminic acid	0.54	10.00	0.57
	1771	L-Carnosine	0.71	10.00	0.72
	1773	O-Cresolphthalein Complexone	0.62	10.00	0.67
	1890	Alloxazine	0.80	5.26	0.58
	2035	5-Bromofuroic acid	0.57	7.14	0.89
	2036	8-Bromoguanosine	0.58	4.34	0.81
	2037	1-Bromohexadecane	0.51	4.00	0.50

MMTV:

	2010	2-Bromo-4,6-dinitroaniline	0.80	0.63	3.57

a chemical to be considered a selective activator. A
minimum inhibition f 3 fold of one promoter and less
than 20% inhibition of the other two promoters was
required for a chemical to be considered a selective
5   inhibitor.   Table 2 gives the names and induction or
inhibition ratios of the lead chemicals identified for
each promoter. Figure 12 illustrates the transcriptional
stimulation and Figure 13 the transcriptional inhibition
observed with some of the lead chemicals. Some of the
10   chemicals activating G-CSF transcription fell into
conspicuous groups of analogs (Table 2; Group A and B).
Although not specifically indicated in Table 2, groups of
homologs   and   analogs   can   also   be   found   for
G-CSF-inhibiting as well as hGH-activating chemicals.
15

To determine the number of lead chemicals, which
reproducibly score as positives in repeated luciferase
assays, two types of experiments were conducted:

20   1) G-CSF lead chemicals #1780, #58, #1783, #1374 were
subjected to 48 independent luciferase assays performed
on the same day. Compounds #58, #1780 and #1374 scored as
positives in every single one of these assays inducing
luciferase expression between 2 and 28-fold (#58), 20 and
25   80-fold (#1780) and 5 and 40-fold (#1374).   Compound
#1783 scored as positive only in half of the 48 repeat
assays probably due to its relatively low induction of
luciferase expression (1.5 to 8-fold).

30   2) All of the 18 lead chemicals inducing luciferase
expression from the MMTV promoter were again subjected to
luciferase assays: 10 chemicals (#453, #519, #562, #765,
#828, #848, #1269, #1316, #1384 and #2148) again induced
luciferase expression between 2.1 and 2.8-fold. Probably
35   due to the relatively low induction level close to the

background of the assay, the other eight lead chemicals
did not repeat on that particular day. The most prominent
lead chemical, #453 (13.3-fold induction in the original
high-throughput assay), was repeated in a total of 3

5   independent assays and consistently induced luciferase
expression from the MMTV promoter between 10 and 35-fold.
Replacing DMSO by methanol to dissolve the chemical did
not affect its ability to activate the MMTV promoter.

10   Aqueous clarified supernatants derived from individual
Actinomyces colonies prepared by standard methods known
to those skilled in the art as well as corresponding
methanol extracts were assayed for modulation of
luciferase expression from three target promoters of

15   interest.

Out of 356 samples tested for modulation of the three
different promoters, 25 samples scored as positives, 7 of
which were promoter-specific. A summary of the obtained

20   results is contained in Figures 52 and 53. Thus high-
throughput screening of fermentation broth samples using
a luciferase expression assay consistently leads to the
discovery of lead samples with the potential to be
developed into novel pharmaceuticals.

25

C. Effects of Lead Chemicals on Endogenous G-CSF mRNA
Levels

Northern blot analysis was used to demonstrate the

30   stimulatory effects of lead chemicals #670 and #1255 on
endogenous G-CSF mRNA levels. As shown in Figure 14,
both OSI #670 and #1255 stimulated production of G-CSF
mRNA, as shown by a G-CSF-specific probe, but not of
actin mRNA, as shown by a b-actin-specific probe. Also

35   shown are the effects of the solvent, DMSO, used to

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dissolve the chemicals and a proteinaceous positive
regulat r, interferon-gamma. From these data it is
concluded that chemicals, which induce luciferase
expression from specific promoters, in plasmids stably
5   integrated into cells, are also capable of stimulating
mRNA production from the corresponding endogenous
promoters without using a reporter system.

To further confirm, that compounds that had been
10   identified in a luciferase expression assay using a
G-CSF specific reporter cell line would be active in
inducing transcription of the endogenous G-CSF gene,
cells from the parental cell line 5637 used to construct
the reporter cell line were incubated with cycloheximide
15   (25 μg/ml), DMSO (0.5%,solvent control) and low, medium
and high concentrations of the compounds 542 (10, 50, 250
μM),1255 (20, 100, 500 μM), 1793(0.25, 1.2, 6.25 μM) and
1904 (20, 100 μM)   for 18 hours. RNA was extracted and
the concentration of G-CSF, GM-CSF and gamma-actin mRNA
20   was determined by the S1 protection method as described
in Materials and Methods. The positions of G-CSF GM-CSF
and gamma-actin specific protected fragments are
indicated (G, GM, A) at the left side of the gel (Figure
15).

25

Interestingly, all four compounds tested increased the
amount of G-CSF mRNA. It is of great interest that at
least two of them, namely #542 and #1793, also increased
the amount of GM-CSF mRNA. Compound #543, a structural
30   analog of #542 showed similar activity.

D. Dose response Analysis of Structurally Related Lead
Chemicals

35   Among the chemicals which specifically activated the

G-CSF prom ter were groups of structural homologs. Three such homologs, #80, #670, and #1780, belong to groups listed in Table 2. These three structurally related chemicals all specifically activated the G-CSF promoter.

5 Dose response graphs obtained with chemicals #80, #670, and #1780 are shown in Figure 16. Although these chemicals all demonstrate large maximal stimulations, it is clear that their potencies, as measured by their ED_{50}'s (concentration of chemical resulting in 50%

10 maximal stimulation), show wide variability (5-70 μM)).

E. Effects of Lead Chemicals on Target Protein Secretion

Two of the most promising lead chemicals (# 542 and

15 #1780), which were shown to stimulate levels of endogenous G-CSF mRNA as well as luciferase expression from the G-CSF promoter/luciferase fusion constructs, were further investigated for their ability to increase G-CSF secretion into the media of 5637 bladder carcinoma

20 cells incubated with the chemicals for 48 hours. The levels of G-CSF in the cell supernatants were determined by a sandwich-antibody assay as described in Materials and Methods (Figure 17).

25 F. Cytotoxicity of Lead Chemical #542

To address the question whether the induction of G-CSF, and GM-CSF transcription by the, compound #542 was a specific effect or rather a phenomenon linked to a

30 potential sensitivity of these promoters to stress exerted by toxic compounds, the concentration dependency of induction of luciferase activity in the reporter cell line G21 was compared to the concentration dependency of inhibition of respiration in FRE cells.

35

Cells were seeded into 96 well microtiterplates (20,000 cells/well) and cultured overnight. Compound #542 was added at various concentrations and the cells were incubated for 6 hours. The MTT-colorimetric assay was

5   carried out on identically treated samples of FRE cells. Induction of luciferase reporter signal (plain line) and on inhibition of respiration (dashed line) are plotted versus the concentrations of compound (Figure 18). The ED50 for induction of luciferase activity differed from

10   the ED50 for inhibition of respiration by a factor of almost 10, which might indicate that compound #542 exerts a specific effect on G- and GM-CSF transcription.

15   G. Luciferase expression assay in yeast

To determine, whether the luciferase expression assay would also be useful to study other than mammalian cells, a yeast expression plasmid carrying the luciferase gene

20   under control of a yeast promoter was constructed and transfected into appropriate yeast cells. Luciferase activity was demonstrated using the procedures described in Materials and Methods and stock cultures were made from position clones.

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What is claimed is:

1.   A method of transcriptionally modulating the
      expression of a homologous gene-of-interest, the
      expression of which is associated with a defined
      physiological or pathological effect within a
5   multicellular organism, which comprises contacting
      a cell, which is capable of expressing the gene,
      with an amount of a molecule effective to
      transcriptionally modulate expression of the gene
      and thereby affect the level of the protein encoded
10   by the gene which is expressed by the cell, which
      molecule (a) does not naturally occur in the cell,
      (b) specifically transcriptionally modulates
      expression of the gene-of-interest, and (c) binds
      to DNA or RNA, or binds to a protein through a
15   domain of such protein which is not a
      ligand-binding domain of a receptor which naturally
      occurs in the cell, the binding of a ligand to
      which ligand-binding domain is normally associated
      with the defined physiological or pathological
20   effect.

2.   A method of claim 1, wherein the molecule does not
      naturally occur in any cell of a higher eucaryotic
      organism.

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3.   A method of claim 1, wherein the molecule does not
      naturally occur in any cell.

4.   A method of claim 1, wherein the molecule is not a
30   naturally occurring molecule.

5.   A method of claim 1, wherein the cell is a cell of

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the multicellular rganism.

6. A method of claim 1, wherein the cell is an animal
    cell.

7. A method of claim 6, wherein the animal cell is a
    human cell.

8. A method of claim 1, wherein the cell is a plant
    cell.

9. A method of claim 1, wherein the cell is a fungal
    cell.

10. A method of claim 1, wherein the cell is a
    protozoan cell.

11. A method of claim 1, wherein the cell is a
    bacterial cell.

12. A method of claim 1, wherein the gene-of-interest
    is a human gene.

13. A method of claim 1, wherein the gene-of-interest
    encodes a hematopoietic protein.

14. A method of claim 13, wherein the hematopoietic
    protein is a colony stimulating factor.

15. A method of claim 14, wherein the colony
    stimulating factor is granulocyte-macrophage colony
    stimulating factor (GM-CSF).

16. A method of claim 14, wherein the colony

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stimulating   factor   is   granulocyte   colony
stimulating factor (G-CSF).

17. A method of claim 14, wherein the colony
stimulating factor is macrophage colony stimulating
factor (M-CSF).

18. A method of claim 13, wherein the hematopoietic
protein is erythropoietin (EPO).

19. A method of claim 1, wherein the gene-of-interest
encodes an interleukin (IL).

20. A method of claim 1, wherein the gene-of-interest
encodes a growth hormone selected from the group
consisting of human, bovine, porcine, avian, ovine,
piscine, and equine growth hormone, and polypeptide
analogs thereof having the biological activity of
the corresponding naturally occurring growth
hormone.

21. A method of claim 1, wherein the gene of interest
is a growth hormone releasing factor.

22. A method of claim 1, wherein the gene-of-interest
is a viral gene.

23. A method of claim 22, wherein the viral gene is a
retroviral gene.

24. A method of claim 23, wherein the retroviral gene
is a gene from the HIV, HTLV-1, or HTLV-2 virus.

25. A method of claim 22, wherein the viral gene is a

gene from a hepatitis virus.

26. A method of claim 22, wherein the viral gene is a gene from a herpes virus.

27. A method of claim 22, wherein the viral gene is a gene from an animal virus.

28. A method of claim 22, wherein the viral gene is a gene from a papilloma virus.

29. A method of claim 22, wherein the viral gene is a gene from a cytomegalovirus.

30. A method of claim 27, wherein the animal virus is a pseudorabies, Marek's, Newcastle's Disease, or IBR virus.

31. A method of claim 1, wherein the gene-of-interest is a plant gene.

32. A method of claim 31, wherein the plant gene encodes an agronomically important trait.

33. A method of claim 32, wherein the agronomically important trait is selected from the group consisting of germination, sprouting, flowering, fruit ripening, salt tolerance, herbicide resistance, pesticide resistance, fungicide resistance, temperature resistance, and growth.

34. A method of claim 1, wherein the gene-of-interest is a protozoan gene.

35. A method of claim 34, wherein the protozoan is

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selected fr m the group consisting of <u>Trypanosoma</u>,
<u>Plasmodium</u>,   <u>Leishmania</u>,   <u>Giardia</u>,   <u>Entamoeba</u>,
<u>Toxoplasma</u>, <u>Babesia</u>, and <u>Cryptosporidiosis</u>.

36. A method of claim 1, wherein the gene-of-interest
     is a helminth gene.

37. A method of claim 1, wherein the gene-of-interest
     is an oncogene.

38. A method of claim 37, wherein the oncogene is the
     <u>phl-abl</u> oncogene.

39. A method of claim 37, wherein the oncogene is
     selected from the group consisting of H-, N-, and
     K-<u>ras</u> oncogenes.

40. A method of claim 37, wherein the oncogene is the
     <u>neu</u> oncogene.

41. A method of claim 37, wherein the oncogene is the
     <u>src</u> oncogene.

42. A method of claim 1, wherein the gene-of-interest
     encodes TGF-ß1.

43. A method of claim 1, wherein the gene-of-interest
     encodes TGF-ß2.

44. A method of claim 1, wherein the gene-of-interest
     encodes TGF-ß3.

45. A method of claim 1, wherein the gene-of-interest
     encodes a naturally occurring receptor.

46. A method of claim 45 wherein the recept r is a
     receptor f r a TGF-ß.

47. A method of claim 45, wherein the receptor is a
     testosterone receptor.

48. A method of claim 45, wherein the receptor is an
     estrogen receptor.

49. A method of claim 45, wherein the receptor is the
     human low density lipoprotein (LDL) receptor.

50. A method of claim 45, wherein the receptor is the
     receptor for a hematopoietic protein selected from
     the group consisting of M-CSF, G-CSF, GM-CSF, and
     EPO.

51. A method of claim 45, wherein the receptor is the
     receptor for an interleukin (IL) selected from the
     group consisting of IL-1, IL-2, IL-3, IL-4, IL-5,
     IL-6, IL-7 and IL-8.

52. A method of claim 45, wherein the receptor is a
     cell surface protein which mediates infection of
     the cell by a virus.

53. A method of claim 52, wherein the virus is selected
     from the group consisting of HIV, HTLV-1, HTLV-2, a
     hepatitis virus, a herpes virus, an animal virus, a
     papilloma virus, a cytomegalovirus, and a
     rhinovirus.

54. A method of claim 1, wherein the receptor which
     naturally occurs in the cell is a testosterone
     receptor.

55. A method f claim 1, wherein the receptor which naturally occurs in th cell is an estrogen receptor.

56. A method of claim 1, wherein in (c) the protein is not the protein encoded by the gene-of-interest.

57. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a reporter gene, which expresses a polypeptide capable of producing a detectable signal, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measureable detectable signal to be produced by the polypeptide expressed by the reporter gene, quantitatively determining the amount of the signal produced, comparing the amount so determined with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable signal produced by the polypeptide expressed by the reporter gene, and thus identifying the molecule as a molecule capable of transcriptionally modulating

the expression f the gene-of-interest.

58. A method of determining whether a molecule not
previously known to be a modulator of protein
biosynthesis is capable of transcriptionally
modulating the expression of a gene-of-interest
which comprises contacting a sample which contains
a predefined number of cells with a predetermined
amount of a molecule to be tested, each such cell
comprising DNA consisting essentially of (i) a
modulatable transcriptional regulatory sequence of
the gene-of-interest, (ii) a promoter of the gene-
of-interest, and (iii) a DNA sequence transcribable
into mRNA coupled to and under the control of, the
promoter, under conditions such that the molecule,
if capable of acting as a transcriptional modulator
of the gene-of-interest, causes a measureable
difference in the amount of mRNA transcribed from
the DNA sequence, quantitatively determining the
amount of the mRNA produced, comparing the amount
so determined with the amount of mRNA detected in
the absence of any molecule being tested or upon
contacting the sample with any other molecule, and
thereby identifying the molecule as one which
causes a change in the amount of detectable mRNA
amount of, and thus identifying the molecule as a
molecule capable of transcriptionally modulating
the expression of the gene-of-interest.

59. A method of any of claims 57 or 58, wherein the
molecule (a) does not naturally occur in the cell,
(b) specifically transcriptionally modulates
expression of the gene-of-interest, and (c) binds
to DNA or RNA or binds to a protein through a
domain of such protein which is not a ligand

binding domain of a r ceptor which naturally occurs
in the cell, the binding of a ligand t which
ligand binding domain is normally associated with a
defined physiological or pathological effect.

60.   A method of any of claims 57 or 58, wherein the
       sample comprises cells in monolayers.

61.   A method of any of claims 57 or 58, wherein the
       sample comprises cells in suspension.

62.   A method of any of claims 57 or 58, wherein the
       cells comprise human, animal, or plant cells.

63.   A method of any of claims 57 or 58, wherein the
       cells are bacterial cells.

64.   A method of any of claims 57 or 58, wherein the
       cells are fungal cells.

65.   A method of any of claims 57 or 58, wherein the
       predefined number of cells is from about 1 to about
       5×10^5 cells.

66.   A method of claim 65, wherein the predefined number
       of cells is from about 2×10^2 to about 5×10^4
       cells.

67.   A method of any of claims 57 or 58, wherein the
       predetermined amount of the molecule to be tested
       is based upon the volume of the sample.

68.   A method of any of claims 57 or 58, wherein the
       predetermined amount is from about 1.0pM to about
       20 μM.

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69. A method of any of claims 57 or 58, wherein the
     predetermined amount is from about 10nM to about
     500µM.

5   70. A method of any of claims 57 or 58, wherein the
          contacting is effected from about 1 hour to about
          24 hours.

71. A method of claim 70, wherein the contacting is
10   effected from about 2 to about 12 hours.

72. A method of any of claims 57 or 58, wherein the
          contacting is effected with more than one
          predetermined amount of the molecule to be tested.

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73. A method of any of claims 57 or 58, wherein the
          molecule to be tested is a purified molecule.

74. A method of any of claims 57 or 58, wherein the
20   modulatable transcriptional regulatory sequence
          comprises a cloned genomic regulatory sequence.

75. A method of any of claims 57 or 58, wherein the DNA
          consists essentially of more than one modulatable
25   transcriptional regulatory sequence.

76. A method of claim 57, wherein the reporter gene is
          inserted downstream of the promoter of the gene-of-
          interest by homologous recombination.

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77. A method of claim 57, wherein the reporter gene
          encodes a luciferase.

78. A method of claim 57, wherein the reporter gene
35   encodes chloramphenicol acetyltransferase.

79. A method of claim 57, wherein the reporter gene encodes β glucuronidase.

80. A method of claim 57, wherein the reporter gene encodes β galactosidase.

81. A method of claim 57, wherein the reporter gene encodes neomycin phosphotransferase.

82. A method of claim 57, wherein the reporter gene encodes guanine xanthine phosphoribosyltransferase.

83. A method of claim 58, wherein mRNA is detected by quantitative polymerase chain reaction.

84. A screening method according to any of claims 57 or 58 which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells, under conditions such that contacting is effected with a predetermined amount of each different molecule to be tested.

85. A screening method of claim 84, wherein the plurality of samples comprises more that about 10^6 samples.

86. A screening method of claim 84, wherein the plurality of samples comprises more than about 6×10^6 samples.

87. A method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes of interest in a panel of such genes which

comprises essentially simultaneously screening the
molecules against each of the genes of interest
according to the method of claim 84.

88. A screening method of any of claims 86 or 87,
     wherein more that about 10^3 samples per week are
     contacted with different molecules.

89. A method for transcriptionally modulating in a
     multicellular organism the expression of a gene-of-
     interest, the expression of which is associated
     with a defined physiological or pathological effect
     in the organism, which comprises administering to
     the organism an amount of a molecule effective to
     transcriptionally modulate expression of the gene
     and thus affect the defined physiological or
     pathological effect, which molecule (a) does not
     naturally occur in the organism, (b) specifically
     transcriptionally modulates expression of the gene-
     of-interest, and (c) binds to DNA or RNA or binds
     to a protein through a domain of such protein which
     is not a ligand binding domain of a receptor which
     naturally occurs in the organism, the binding of a
     ligand to which ligand binding domain is normally
     associated with the defined physiological or
     pathological effect.

90. A method of claim 89, wherein the multicellular
     organism is a human being.

91. A method of claim 89, wherein the multicellular
     organism is an animal.

92. A method of claim 89, wherein the multicellular organism is a plant.

93. A method of claim 89, wherein the defined pathological effect is a disorder and modulated expression of the gene-of-interest is associated with amelioration of the disorder.

94. A method of claim 90, wherein the defined pathological effect is a disorder selected from the group consisting of cancer, a hematopoietic dysfunction, diabetes, tissue inflammation, atherosclerosis, viral infections, dysfunctions of memory or learning, and dysfunctions in a cholesterol or other metabolic pathway.

95. A method of claim 91, wherein the defined physiological effect is growth and the organism is an animal such as a man, a cow, a pig, a bird, a fish, a sheep or a horse.

96. A method of claim 92, wherein the defined physiological or pathological effect is an agronomically important trait.

97. A method of claim 90 or 91, wherein the administering comprises topical contact.

98. A method of claim 90 or 91, wherein the administering comprises oral, transdermal, intravenous, intramuscular or subcutaneous administration.

99. A method of any of claims 57, 58, or 89, wherein the gene-of-interest encodes a naturally occurring

receptor.

100. A method of claim 99, wherein the receptor is a
testosterone receptor.

101. A method of claim 99, wherein the receptor is an
estrogen receptor.

102. A method of any of claims 57, 58, or 89, wherein
the receptor which naturally occurs in the cell is
a testosterone receptor.

103. A method of any of claims 57, 58, or 89, wherein
the receptor which naturally occurs in the cell is
an estrogen receptor.

104. A method of any of claims 57, 58, or 89, wherein
the gene-of-interest encodes a TGF-ß receptor.

105. A method of any of claims 57, 58, or 89, wherein
the gene-of-interest encodes TGF-ß1.

106. A method of any of claims 57, 58, or 89, wherein
the gene-of-interest encodes TGF-ß2.

107. A method of any of claims 57, 58, or 89, wherein
the gene-of-interest encodes TGF-ß-3.

108. A method of any of claims 57, 58, or 89, wherein
the gene-of-interest encodes an oncogene.

109. A method of claim 108, wherein the oncogene is the
neu oncogene.

110. A method of claim 108, wherein the oncogene is

selected from the group consisting f H-, N-, and
K-<u>ras</u> oncogenes.

111. A method for enhancing the expression of human
growth hormone by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing, human
growth hormone, comprising contacting the cell with
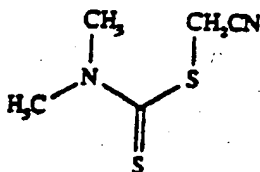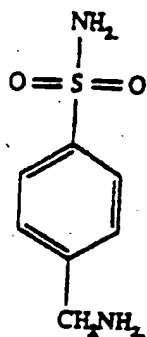an amount of a molecule having the structure:
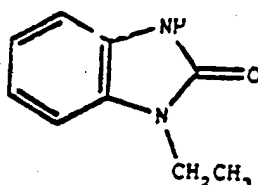
effective to enhance the expression of human
growth hormone by the cell.

112. A method for enhancing the expression of human
growth hormone by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing
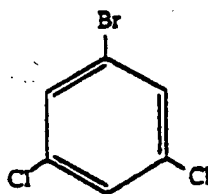contacting the cell with an amount of a molecule
having the structure:

_134_
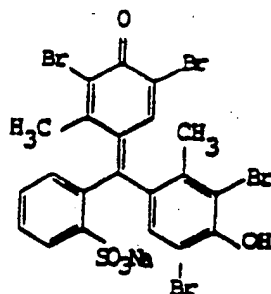
effective to enhance the expressi n of human
growth hormone by the cell.

113. A method for enhancing the expression of human
     growth hormone by a cell which (i) comprises DNA
     encoding, and (ii) is capable of expressing, human
     growth hormone, comprising contacting the cell with
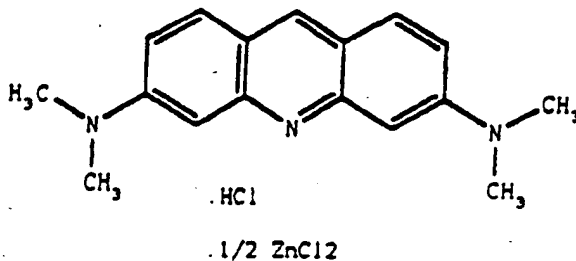   an amount of a molecule having the structure:
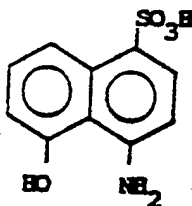
effective to enhance the expression of human
growth hormone by the cell.

114. A method for enhancing the expression of human
     growth hormone by a cell which (i) comprises DNA
     encoding, and (ii) is capable of expressing, human
     growth hormone, comprising contacting the cell with
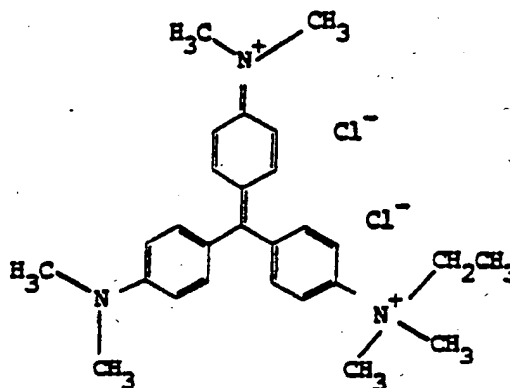   an amount of a molecule having the structure:

effective t enhance th expression of human growth
hormone by the cell.

136

115. A method for enhancing the expression of human
     growth hormone by a cell which (i) comprises DNA
     encoding, and (ii) is capable of expressing, human
5   growth hormone, comprising contacting the cell with
     an amount of a molecule having the structure:

$$-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-NHCH_2CH_2CH_2\overset{CH_3}{\underset{CH_3}{\overset{|}{N+}}}CH_3 \quad \text{or} \quad -\overset{-}{O}-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-OCH_3,$$

     wherein R is
15   effective to enhance the expression of human
     growth hormone by the cell.
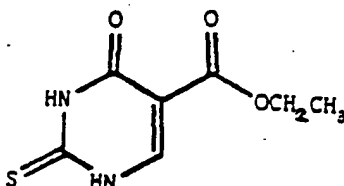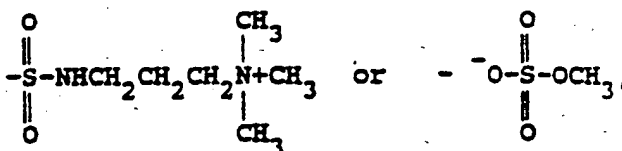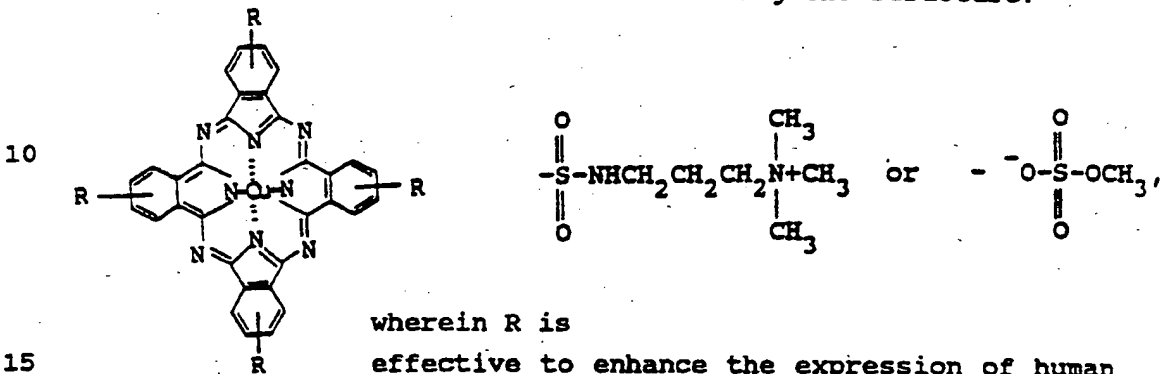
116. A method for enhancing the expression of G-CSF by a
     cell which (i) is capable of expressing, G-CSF,
20   comprising contacting the cell with an amount of a
     molecule having the structure:
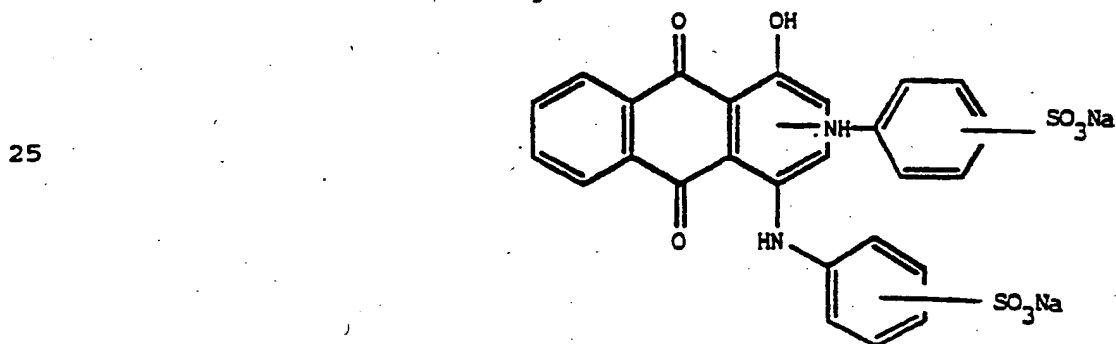
30   effective to enhance the expression of G-CSF
     by the cell.

117. A method for enhancing the expression f G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
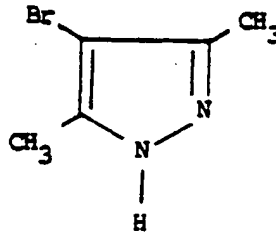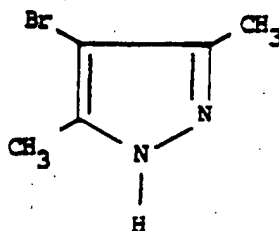the cell with an amount of molecule having the
structure:
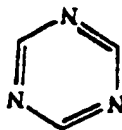
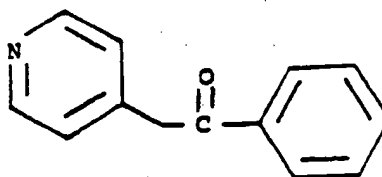effective to enhance the expression of G-CSF
by the cell.

118. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:
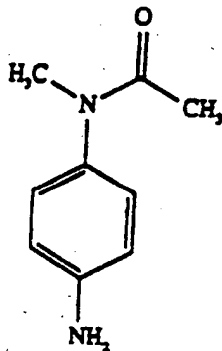
effective to enhance the expression of G-CSF
by the cell.

119. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
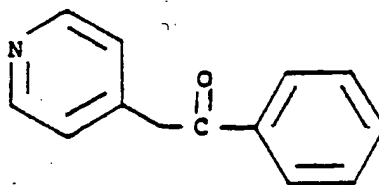the cell with an amount of a molecule having the
structure:
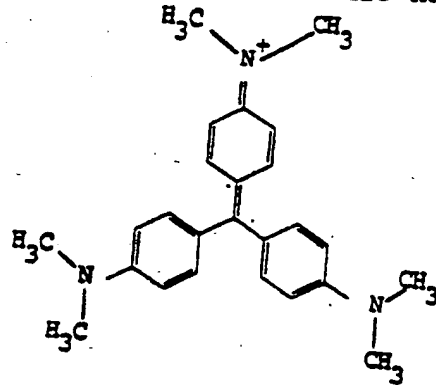
effective to enhance the expression of G-CSF
by the cell.

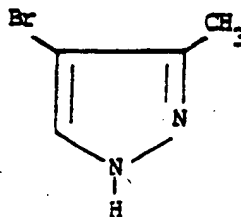120. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:
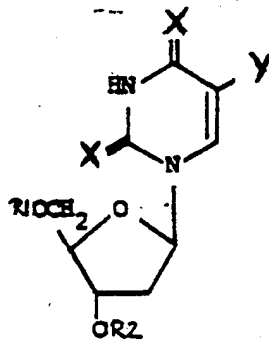
wherein R1 is hydrogen or a lower alkyl group,
wherein R2 is hydrogen or a lower alkyl group,
wherein X is oxygen or $-NH_2$,
wherein Y is bromine, fluorine, chlorine, or
iodine, and
which molecule is effective to enhance the
expression of G-CSF by the cell.

121. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

effective to enhance the expression of G-CSF
by the cell.

122. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

140

$$O = S = O$$ with NH_2 above S, benzene ring below, CH_2NH_2 at bottom

effective to enhance the expression of G-CSF
by the cell.

123. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

Br, benzene ring with Cl and Cl at bottom positions

effective to enhance the expression of G-CSF
by the cell.

124. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

141

.HCl

.1/2 ZnCl2

effective to enhance the expression of G-CSF
by the cell.

125. A method of decreasing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
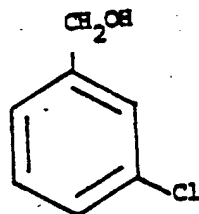capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

effective to decrease the expression of G-CSF
by the human being.

126. A method for decreasing the expression of G-CSF by
a cell which (i) comprises DNA encoding, and (ii)

142

is capable of expressing, G-CSF, comprising
contacting the cell with an amount of a molecule
having the structure:

5

10

effective to decrease the expression of G-CSF
by the human being.

127. A method of decreasing the formation of neutrophils
     and affecting the metabolic functions of
     neutrophils in a human being who (i) comprises DNA
     encoding, and (ii) is capable of expressing, G-CSF,
     comprising administering to the human being an
     amount of a molecule having the structure:
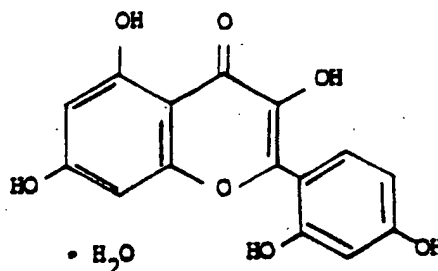

     effective to decrease expression of G-CSF by,
     and thus decrease the formation and effect the
     metabolic functions of neutrophils in, the
     human being.

128. A method of decreasing the expression of G-CSF by a
     cell which (i) comprises DNA encoding, and (ii) is
     capable of expressing, G-CSF, comprising contacting
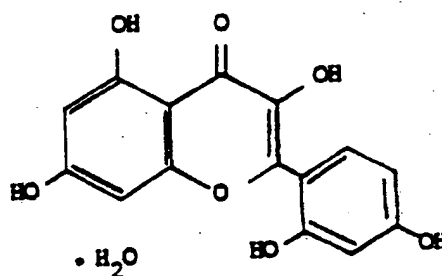   the cell with an amount of a molecule having the
     structure:

     effective to decrease the expression of G-CSF

144

by the human being.

129. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

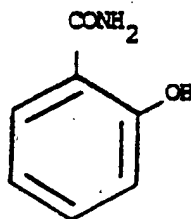effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

130. A method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising contacting the cell with an amount of a molecule having the structure:
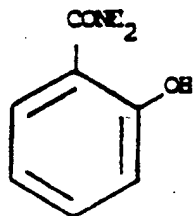
145

effective to decrease the expression of G-CSF
by the human being.

131. A method of decreasing the formation of neutrophils
and affecting the metabolic functions of
neutrophils in a human being who (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an
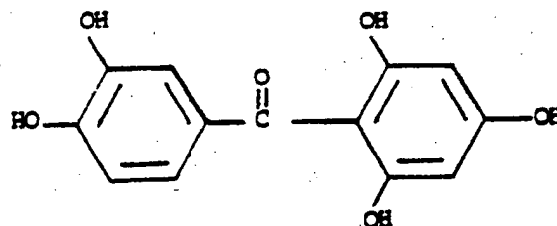amount of a molecule having the structure:
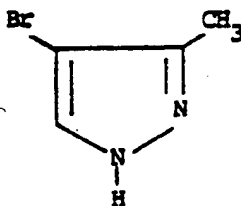
effective to decrease expression of G-CSF by,
and thus decrease the formation and effect the
metabolic functions of neutrophils in, the
human being.

132. A method of decreasing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of xpressing, G-CSF, comprising contacting

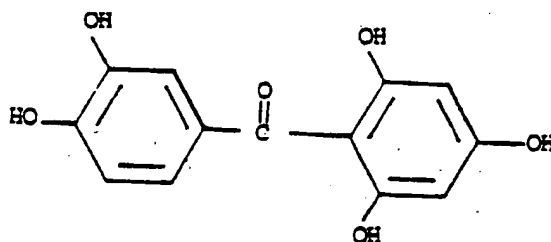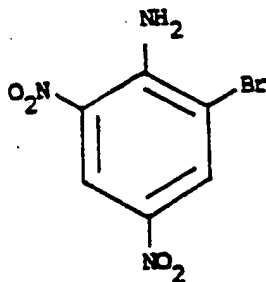the cell with an amount of a molecul having the
structure:
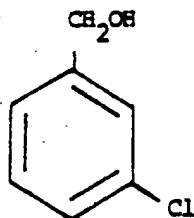
effective to decrease the expression of G-CSF by
the human being.

133. A method of decreasing the formation of neutrophils
      and affecting the metabolic functions of
      neutrophils in a human being who (i) comprises DNA
      encoding, and (ii) is capable of expressing, G-CSF,
      comprising administering to the human being an
      amount of a molecule having the structure:

effective to decrease expression of G-CSF by, and
thus decrease the formation and effect the
metabolic functions of neutrophils in, the human
being.

134. A method of decreasing the expression of G-CSF by a
      cell which (i) comprises DNA encoding, and (ii) is
      capable of expressing, G-CSF, comprising contacting

147

the cell with an amount of a molecule having the
structure:

• H$_2$O

effective to decrease the expression of G-CSF
by the human being.

135. A method of decreasing the formation of neutrophils
and affecting the metabolic functions of
neutrophils in a human being who (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an
amount of a molecule having the structure:

• H$_2$O

effective to decrease expression of G-CSF by,
and thus decrease the formation and effect the
metabolic functions of neutrophils in, the
human being.

136. A method f decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising contacting the cell with an amount of a molecule having the structure:

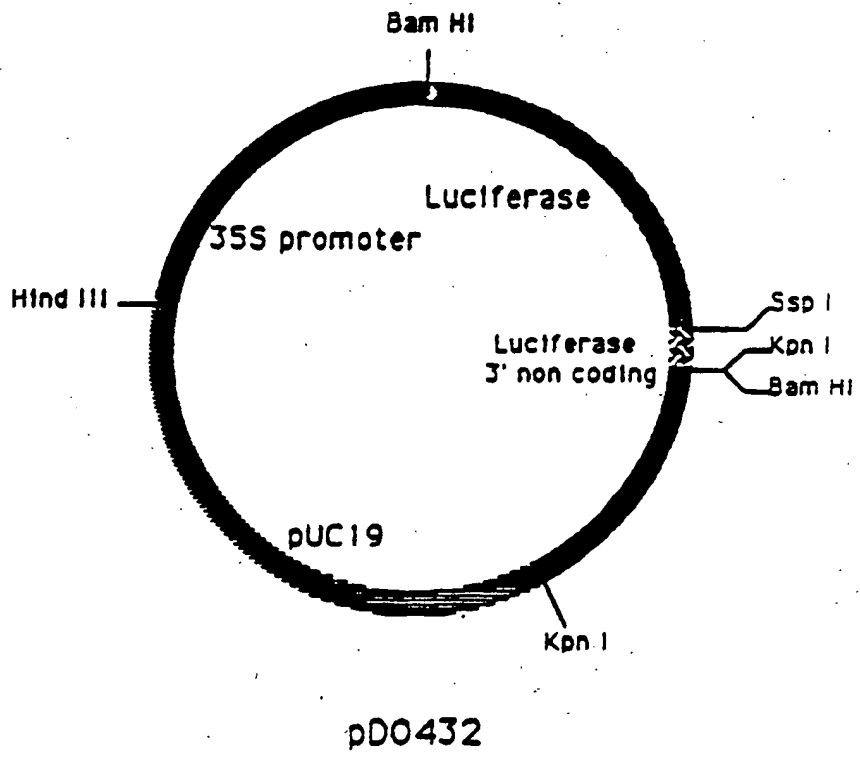effective to decrease the expression of G-CSF by the human being.

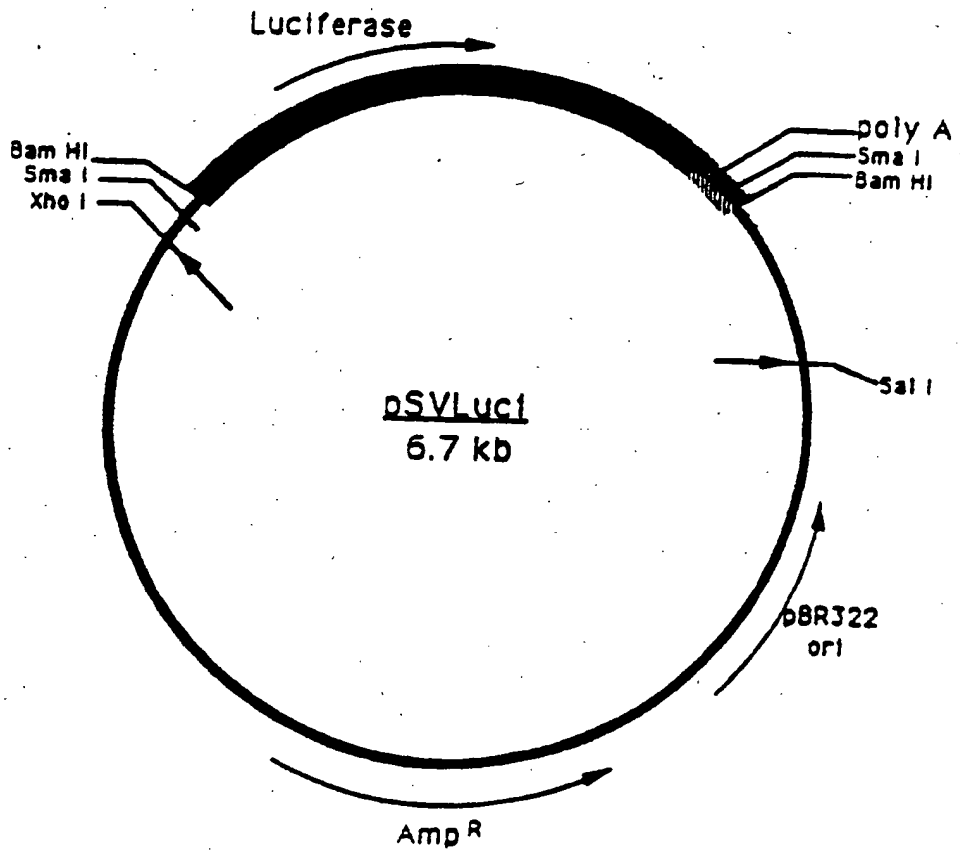137. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

149

effective to decrease expression of G-CSF by,
and thus decrease the formation and effect the
metabolic functions of neutrophils in, the
human being.

138. A method of decreasing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

effective to decrease the expression of G-CSF
by the human being.

139. A method of decreasing the formation of neutrophils
     and affecting the metabolic functions of
     neutrophils in a human being who (i) comprises DNA
     encoding, and (ii) is capable of expressing, G-CSF,
     comprising administering to the human being an
     amount of a molecule having the structure:

effective to decrease expression of G-CSF by,
and thus decrease the formation and effect the
metabolic functions of neutrophils in, the
human being.

140. A method of decreasing the expression of G-CSF by a
     cell which (i) comprises DNA encoding, and (ii) is
     capable of expressing, G-CSF, comprising contacting
     the cell with an amount of a molecule having the
     structure:

effective to decrease the expression of G-CSF
by the human being.

5

1/18

Figure 1. pDO432

pDO432

Figure 2. pSVLuci

Figure 3. pMLuci
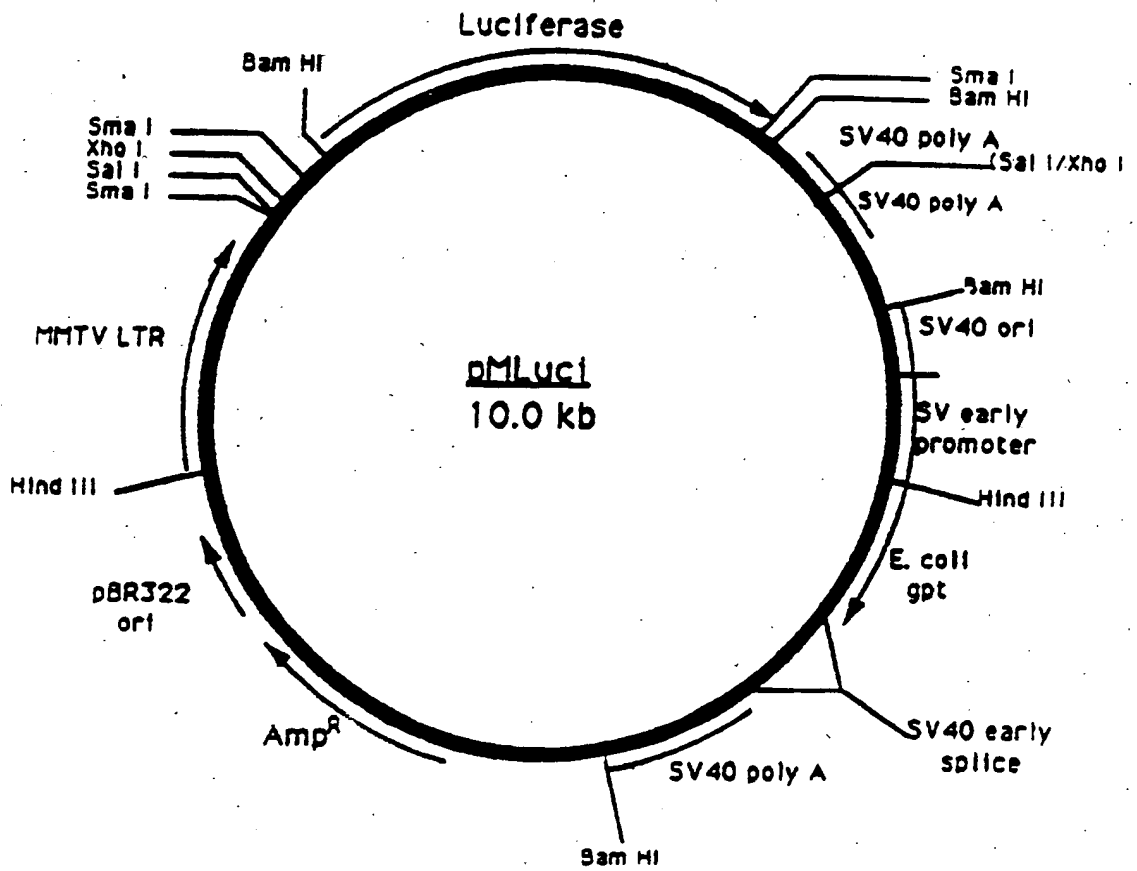
Figure 4. pUXLuc1
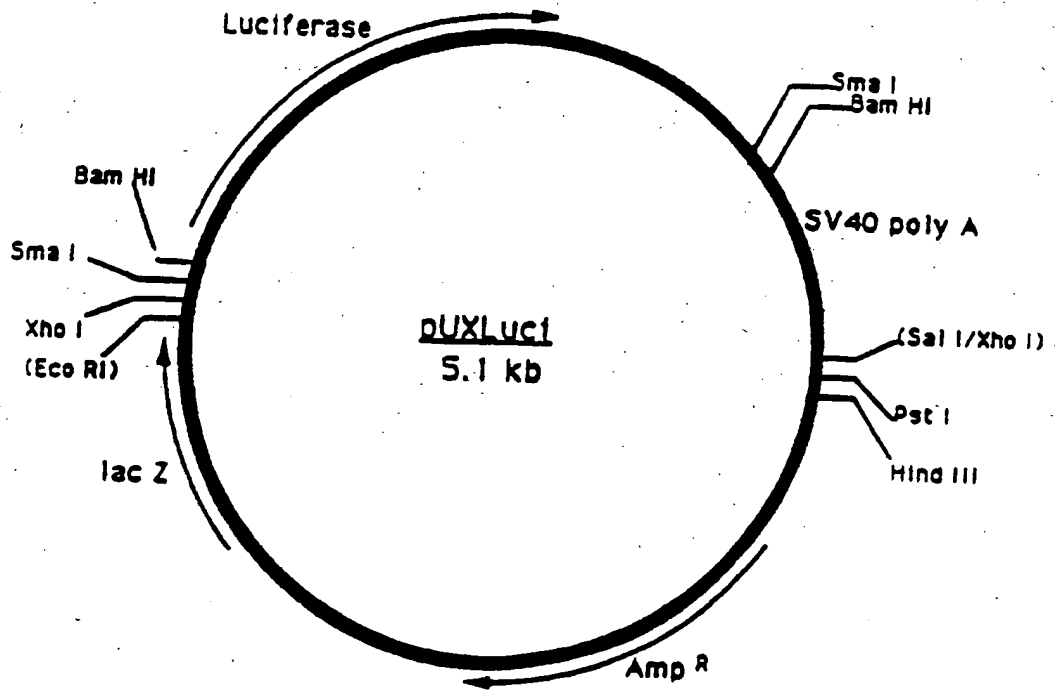
Figure 5. phGH-CAT

Figure 6. phGH-Luci

pUXLuci

hGH promoter

Xba I
Sal I
Bam HI

Luciferase

phGH:luci
5650 bp
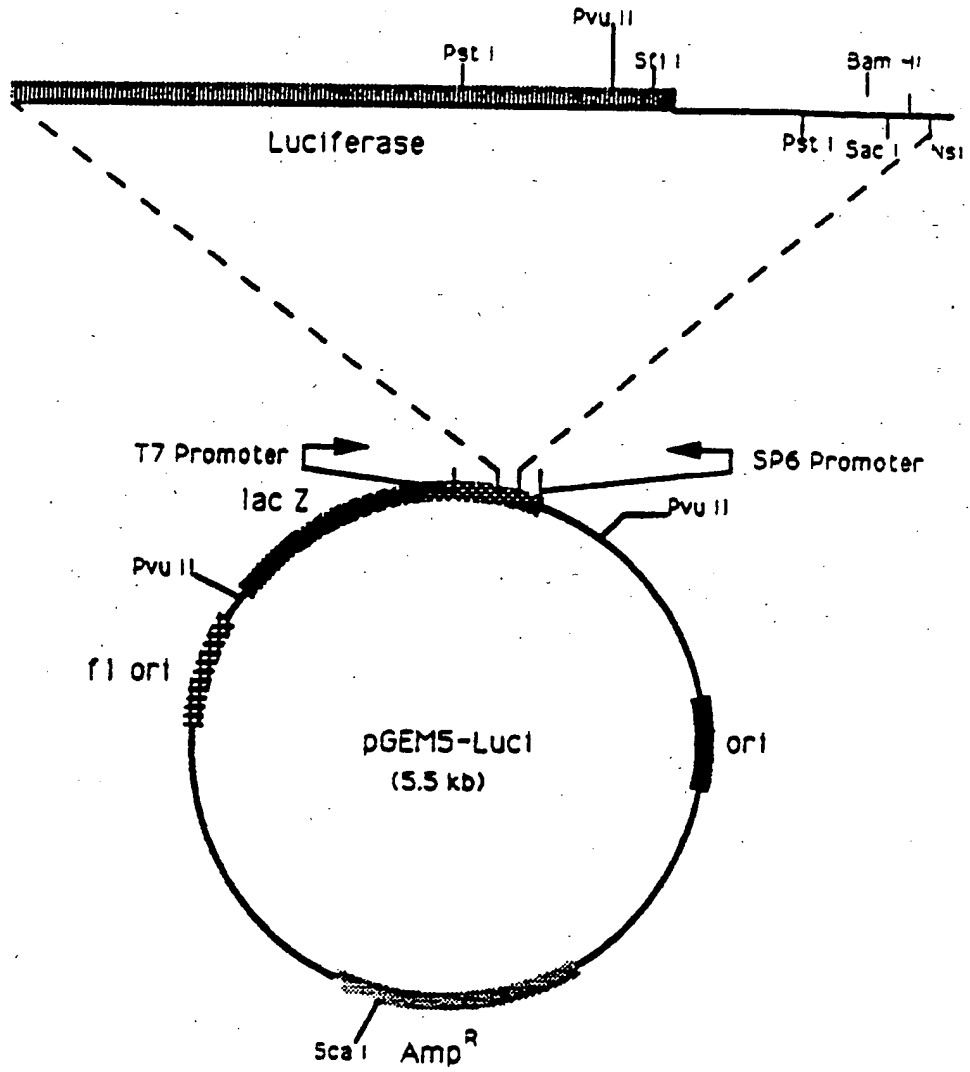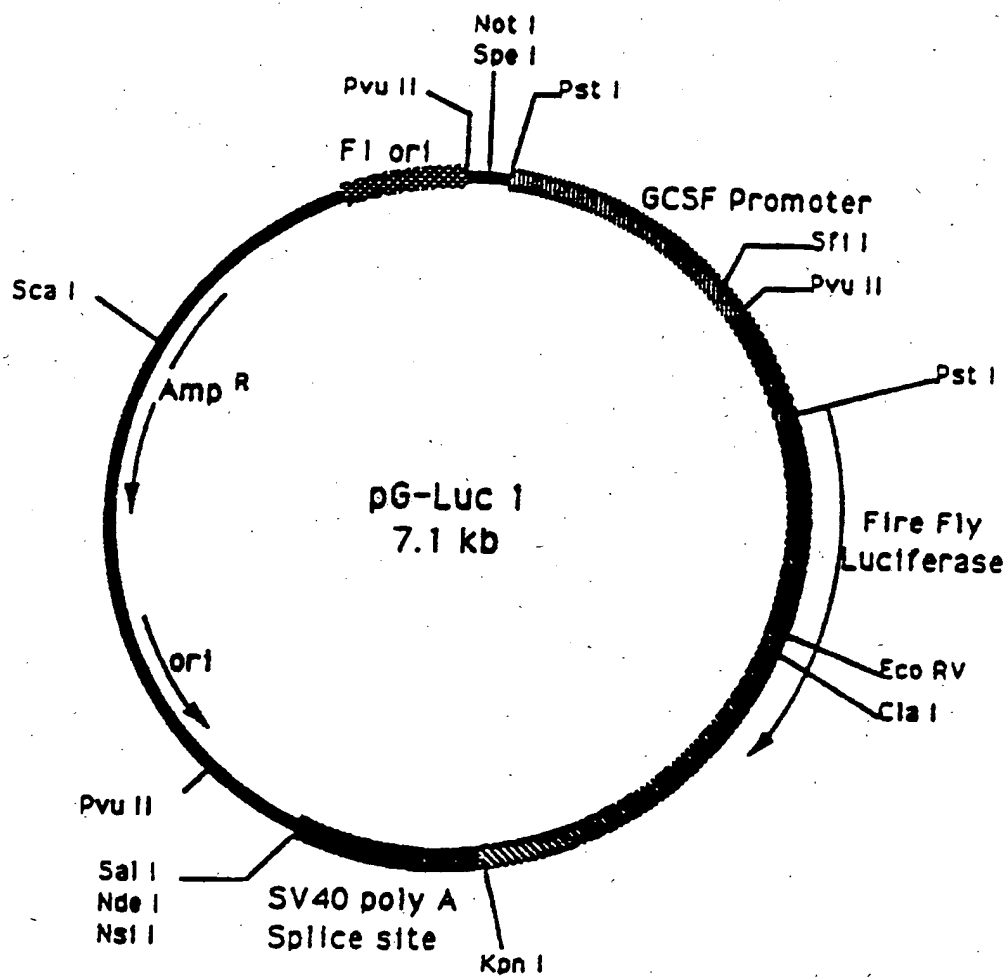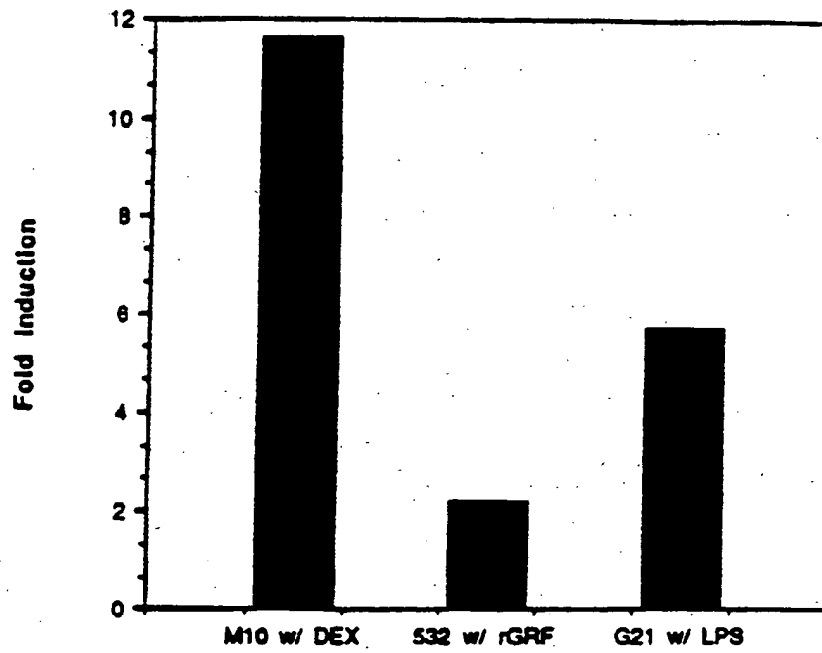
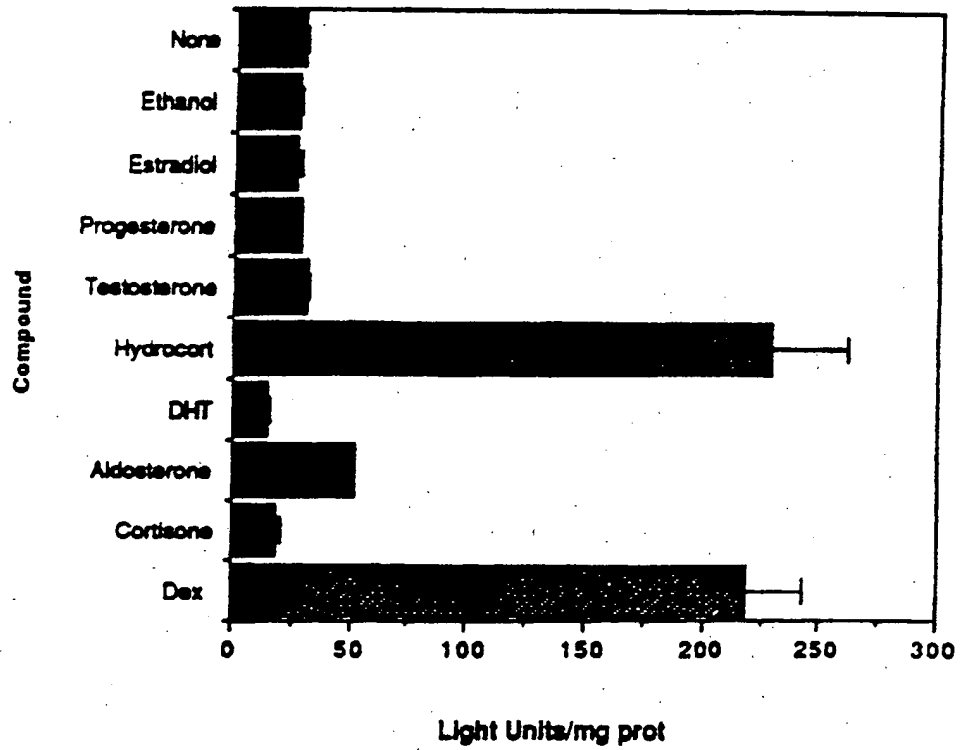SV40 late poly A

ori

Amp^R
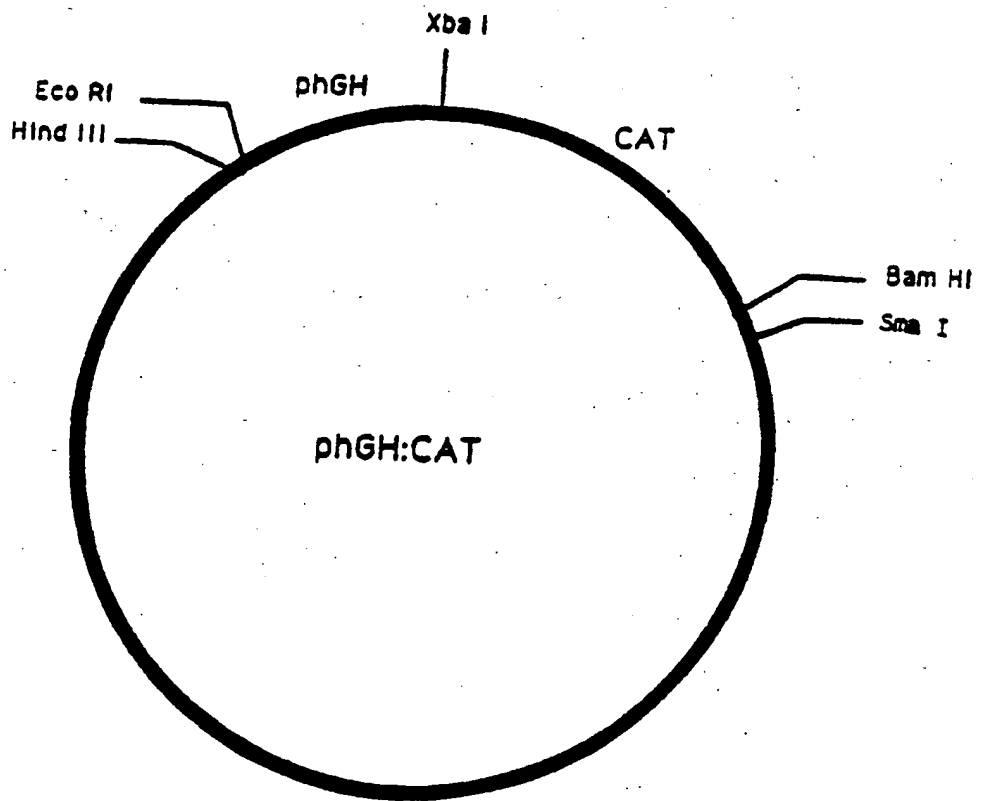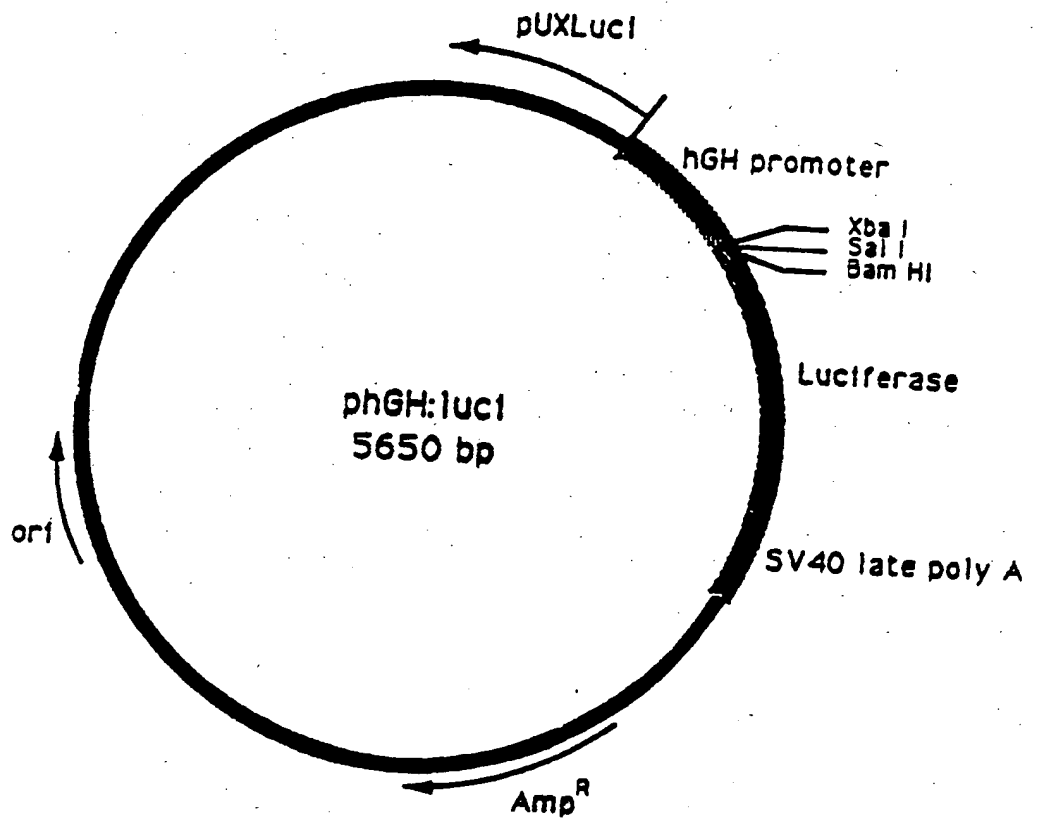
Figure 7. pJM710
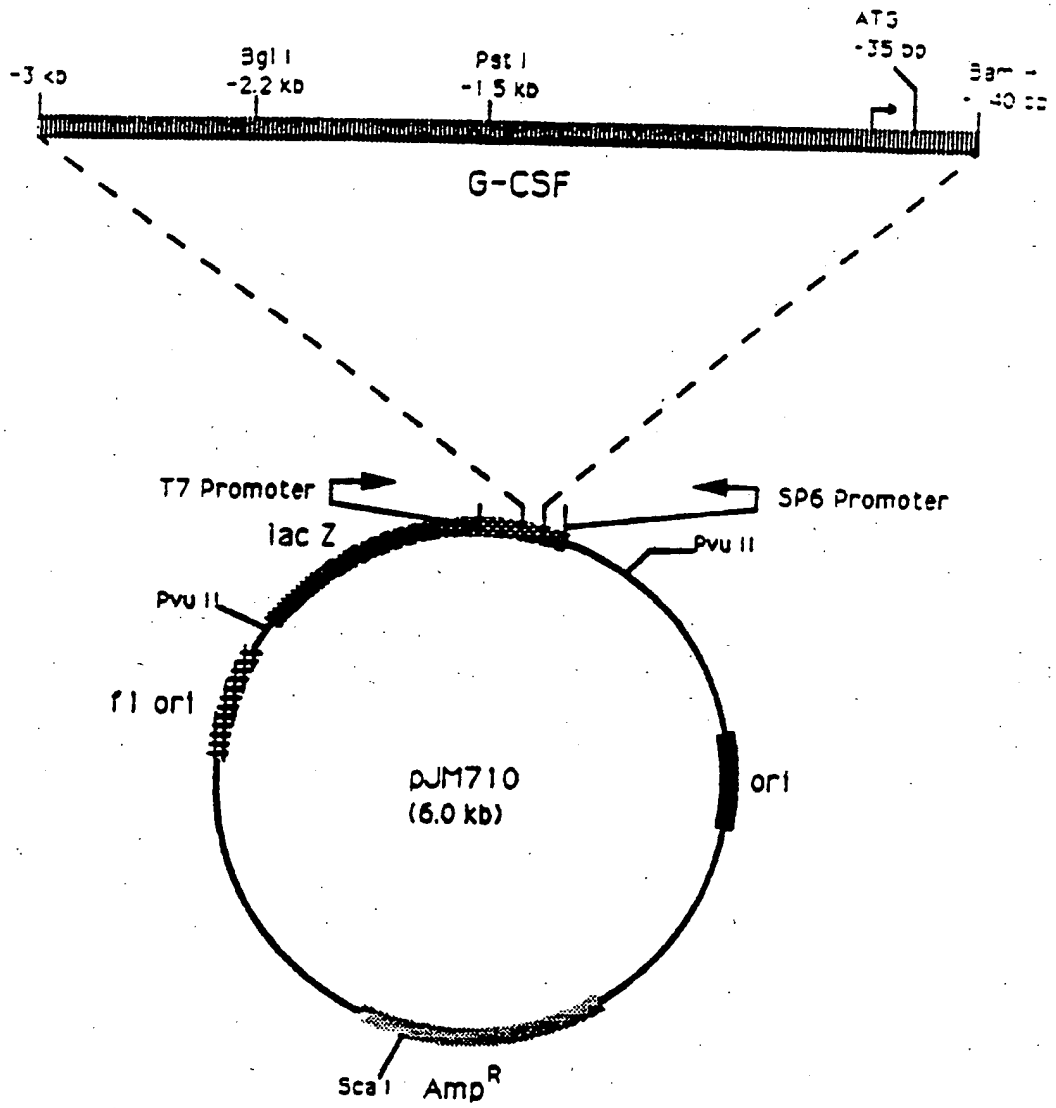
Figure 8. pGEM5-Luci

Figure 9. pG-Luc 1

Figure 10. Response of Reporter Cell Clones To Known Inducers

Figure 11 . Effects of Steroids on Clone M10

Figure 12

EXAMPLES OF PRIMARY SCREEN LEAD CHEMICALS—
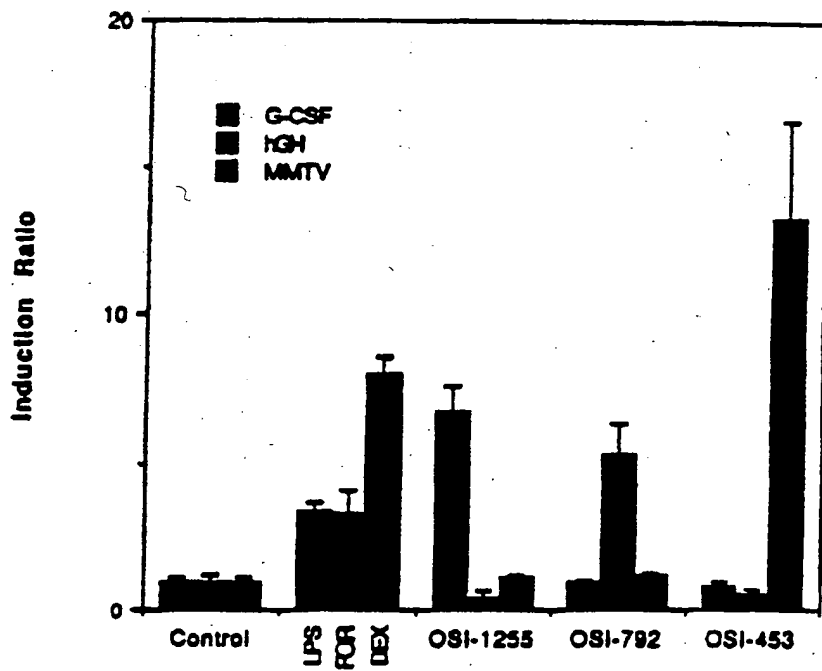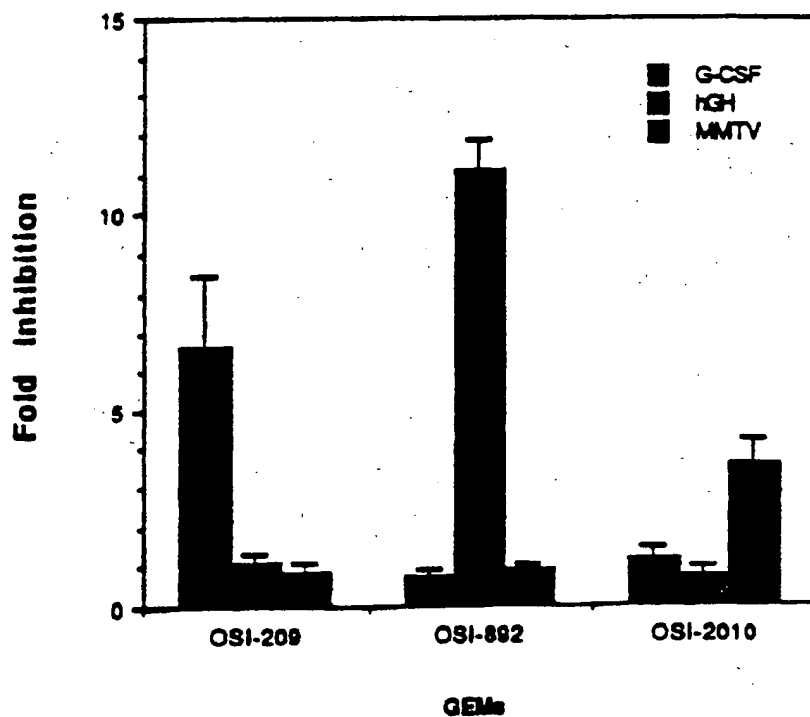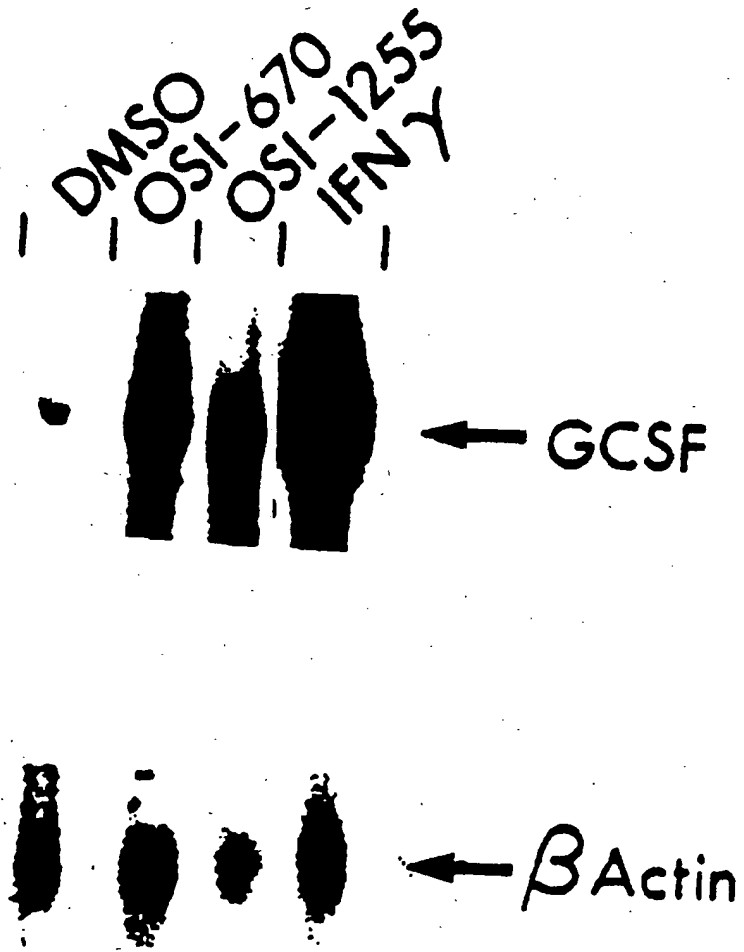SPECIFIC TRANSCRIPTIONAL INDUCERS

Figure 13

EXAMPLES OF PRIMARY SCREEN LEAD CHEMICALS
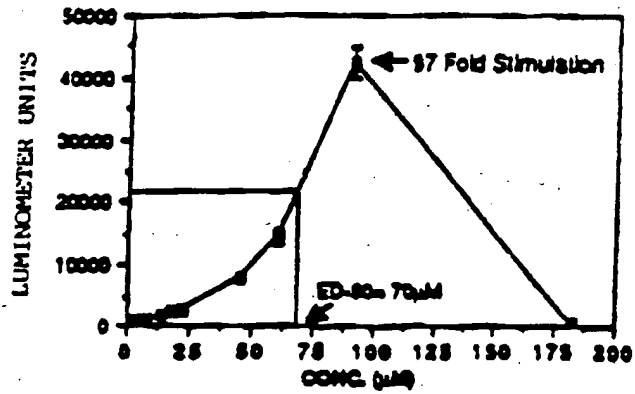SPECIFIC TRANSCRIPTIONAL INHIBITORS
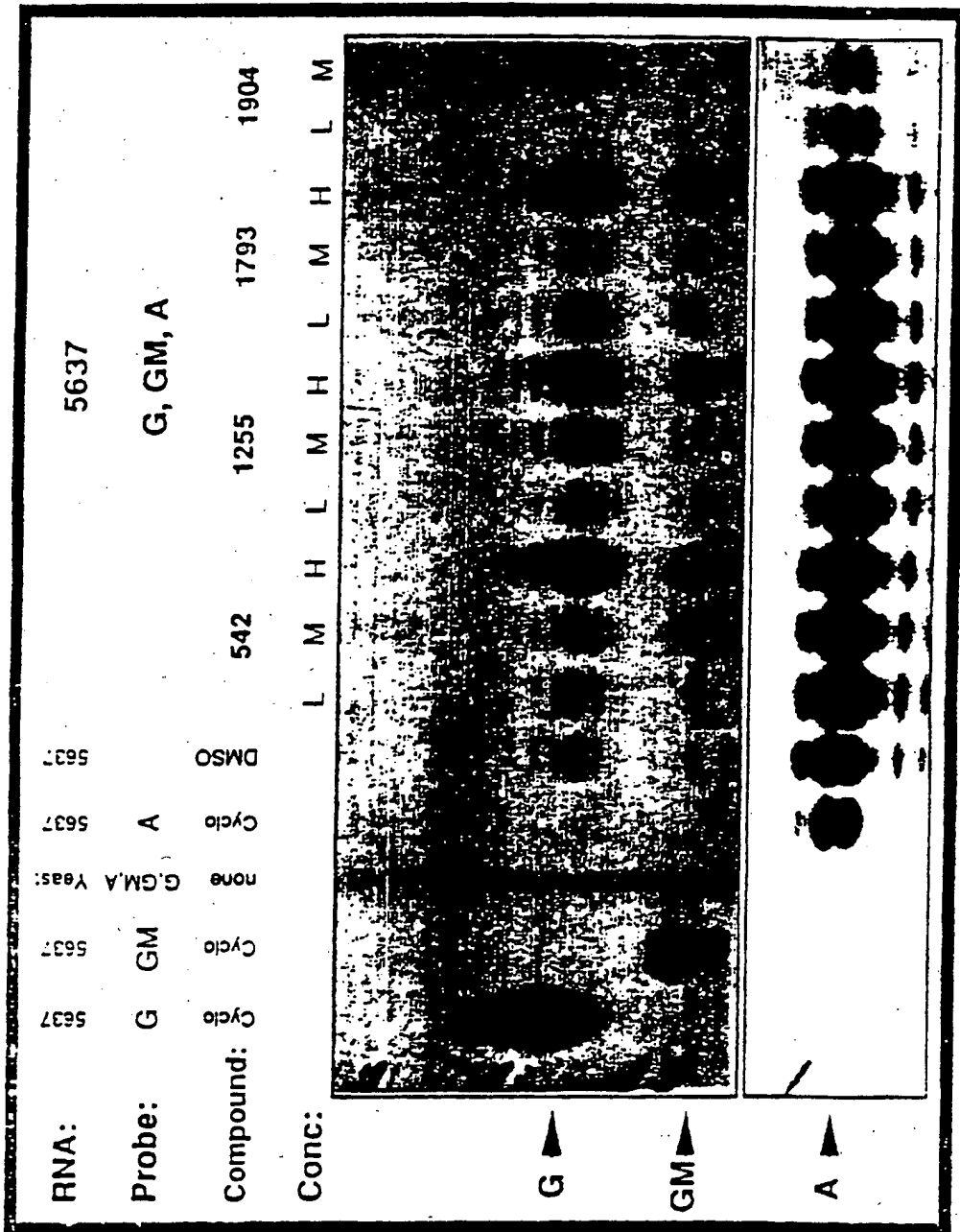
Figure 14

Figure 15

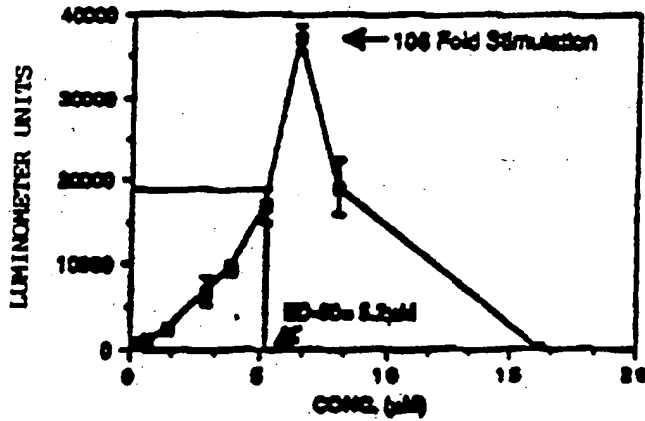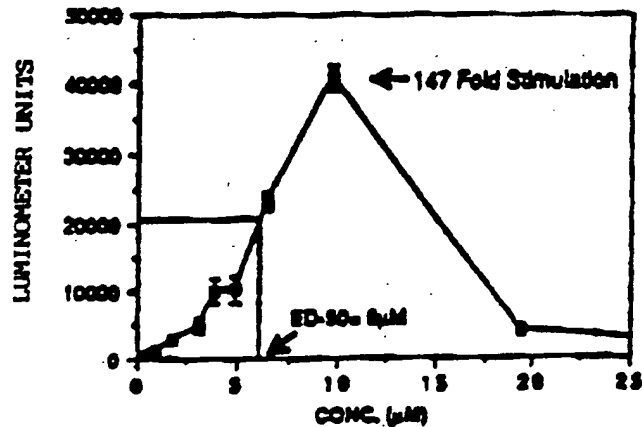## Figure 16

DOSE RESPONSE ANALYSIS OF CMPD 670

DOSE RESPONSE ANALYSIS OF CMPD 1780

DOSE RESPONSE ANALYSIS OF CMPD 80

Figure 17

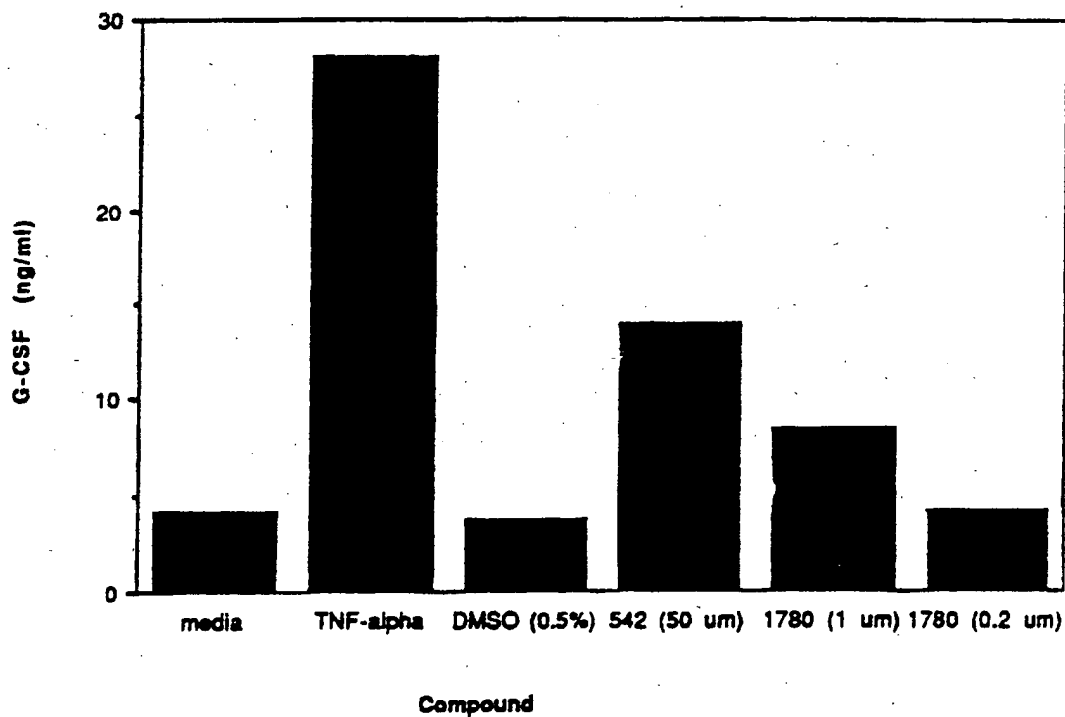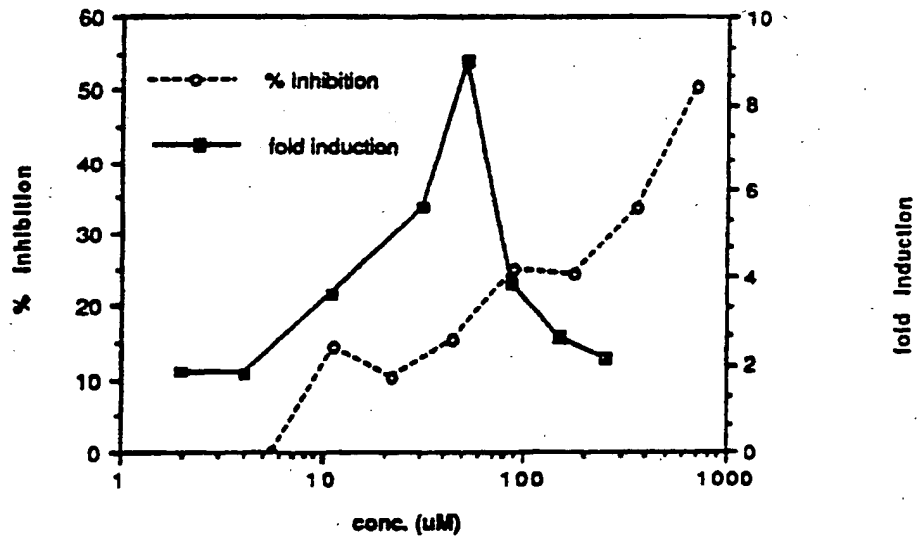G-CSF concentration in cell supernatant after 48 hours incubation

Compound

Figure 18

542 - promoter stimulation versus respiratory inhibition

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04021

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(5):   C12P 21/00,21/02; C12N 7/04,5/00,15/00,1/38; C12Q 1/66
USA: 435/69.5,69.62,69.4,70.1,236,240.2,172.1,172.2,172.3,244

II. FIELDS SEARCHED		
Minimum Documentation Searched [4]		
Classification System		Classification Symbols
US		435/8,69.4,69.5,69.62,70.1,172.1,172.2,172.3,236,240.2,244

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]
U.S. Patent and Trademark Office Automated patent System DIALOG- BIOSIS PREVIEWS, CHINESE PATENT ABSTRACTS, CLAIMS™/U.S. PATENTS, INPADOC/FAMILY AND LEGAL STATUS, WORLD PATENTS INDEX (SEE ATTACHMENT)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]		
Category [*]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	US, A, 4,738,922 (Haseltine et al.) 19 April 1988. See col. 1-10 and the examples.	1-6 1-7,12
X Y	US, A, 4,740,461 (Kaufman) 26 April 1988. See col. 1-16 and the examples.	1-6 1-7,12
X Y	EP, A, 0,117,058 (Levinson et al.) 29 August 1984. See at least the abstract and pages 5-18.	1-6 1-7,12
X Y	Mol. Cell. Biol. Volume 7, No.6, issued June 1987 (Angel et al.) "12-0-tetradecanoyl phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region pages 2256-2266. See the abstract and figures.	1-7 1-7,12

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
17 October 1990	02 JAN 1991
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	*Christopher Low*

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Proc. Natl. Acad. Sci. USA, Volume 83, issued May 1986 (Kaushansky et al.) "Genomic cloning, characterization, multilineage growth-promoting activity of human granulocyte-macrophage colony-stimulating factor". Pages 3101-3105. See Figure 2, and page 3105. (See Attachment)	1-7,12-15

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers .... because they relate to subject matter [1] not required to be searched by this Authority, namely:

2.☐ Claim numbers .... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3.☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

(See Attachment)

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-7 and 12-19

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 4-90)

Y   EMBO J. Volume 6, No. 9, issued September 1987   1-7,12-14,
      (Ladner et al.) "Human CSF-1: gene structure and   17
      alternative splicing of mRNA". pages 2693-2698
      See at least page 2693, 2696-2997.

Y   EMBO J. Volume 6, No. 4, issued April 1987   1-7, 12
      (Lefevre et al.) "Tissue-specific expression of
      the human growth hormone gene is conferred in
      part by the binding of a specific trans-acting
      factor". pages 971-981. See at least page 971.

Y   Proc. Natl. Acad. Sci. USA. Volume 82, issued   1-7,12,13
      November 1985 (Lin et al.) "Cloning and   18
      expression of the human erythropoietin gene".
      pages 7580-7584. See entire document.

Y   EMBO J. Volume 5, No. 3, issued March 1986,   1-7,12-14
      (Nagata et al.) "The chromosomal gene structure   16
      and two mRNAs for human granulocyte colony-
      stimulating factor. pages 575-581. See at least
      the abstract.

Y   Cell Volume 47, issued 10 October 1986, (Yang   1-7,12-14,
      et al.) "Human IL-3 (multi-CSF): Identification 17,19
      by expression cloning of a novel hematopoietic
      growth factor related to murine IL-3. pages
      3-10. See Figure 4, and page 6.

Y   de Serres et al., "Chemial Mutagens. Principles 1-7,12-19
      and methods for their detection", published
      1980, Plenum Press, New York. pages 331, and
      365-473. See at least pages 331, 367-369, 377.

Y   Science Volume 227, issued 15 March 1985,   1-7,12-19
      (Engebrecht et al.) "Measuring gene expression
      with light". pages 1345-1347. See entire
      document.

Y   Science Volume 236, issued 05 June 1987,   1-7,12-19
      (Maniatis et al.) "Regulation of inducible
      tissue-specific gene expression. pages 1237-1245
      See pages 1237, 1239, 1240, 1243.

Y   Molec. Cell. Biol. Volume 7, No. 2, issued   1-7, 12-19
      February 1987, (de Wet et al.) "Firefly
      luciferase gene: Structure and expression in
      mammalian cells". pages 725-737. See the
      abstract, Fig. 1 and 3, pages 729-734.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Search Terms:

mammal, toxicity, toxic, chemial, chemicals, carcinogen, carcinogenic, carcinogen, oncogenic, oncogen, transcript